TITLE OF THE INVENTION

Recombinant *P. falciparum* Merozoite Protein-1_{42} Vaccine

5   This application claims the benefit of priority under 35 U.S.C. §119(e) from U.S. application serial no. 60/264,535 filed on January 26, 2001, still pending, and U.S. provisional application filed on October 26, 2001, still pending.

10   **INTRODUCTION**

Plasmodium falciparum is the leading cause of malaria morbidity and mortality. The World Health Organization estimates that approximately 200 million cases of malaria are reported yearly, with 3 million

15   deaths (World Health Organization, 1997, Wkly. Epidemiol. Rec. 72:269-276). Although, in the past, efforts have been made to develop effective controls against the mosquito vector using aggressive applications of pesticides, these efforts ultimately

20   led to the development of pesticide resistance. Similarly, efforts at treatment of the disease through anti-parasitic drugs led to parasite drug-resistance. As the anti-vector and anti-parasite approaches failed, efforts became focused on malaria vaccine

25   development as an effective and inexpensive alternative approach.

However, the complex parasitic life cycle has further confounded the efforts to develop efficacious vaccines for malaria. The parasite's life cycle is

30   divided between the mosquito-insect host and the human host. While in the human host, it passes through several developmental stages in different organellar environments, i.e. the liver stage, the red blood stage. Although conceptually simple, in reality the

problems that must be considered when designing
subunit vaccines for malaria are great. Antigen
diversity is a characteristic that must be taken into
account and includes a high degree of developmental
5   stage specificity, antigenic variation and antigen
polymorphism. Vaccine candidates have been identified
from each of the parasite's developmental stages. The
major merozoite surface protein-1, MSP-1, is among the
leading erythrocytic stage vaccine candidates (Diggs,
10   et al, 1993, Parasitol. Today 9: 300-302). The
objective of erythrocytic stage vaccines is to
diminish the level of parasitemia in the bloodstream
and thus reduce the severity of disease.

Although the MSP-1 molecule has been studied
15   extensively, its function is not fully understood.
There is evidence that MSP-1 binds to erythrocytes and
may have a role in erythrocyte invasion (Perkins and
Rocco, 1988, J. Immunol. 141, 3190-3196; Holder, A.
A., 1994, Parasitology 108 (Suppl.) S5-18).

20   MSP-1 is secreted as a membrane-anchored (Haldar
et al., 1985, J. Biol. Chem. 260, 4969-4974) 195 kDa
precursor that is proteolytically processed to
products with nominal molecular masses of 83, 28-30,
38-45, and 42 kDa during merozoite development (Holder
25   and Freem,an, 1984, Phils Trans R. Soc. Lond B. Bio.
Sci. 307, 171-177; Lyon et al., 1987, J. Immunol, 138,
895-901; Holder et al., 1987, Parasitology 94, 199-
208). These protein fragments form a non-covalent
complex on the surface of merozoites (McBride and
30   Heidrich, 1987, Parasitology 23, 71-84; Lyon, et al.,
1987, supra) that remain attached to the merozoite
surface through the C-terminal 42 kDa fragment (MSP-
1_{42}). At the time of erythrocyte invasion MSP-1_{42} is
processed further to a 33 kDa fragment and a 19 kDa C-
35   terminal fragment (MSP-1_{19}) (Blackman, et al., 1991,

Mol. Biochem. Parasitol. 49, 35-44) which is bound to the merozoite surface through an N-glycosylphosphatidyl inositol anchor (GPI) (Haldar, et al., 1985, supra). This second proteolytic cleavage event results in the shedding of the non-covalent associated protein complex from the merozoite surface during invasion. During the invasion process, MSP-1$_{19}$ is present on ring forms in the newly invaded erythrocyte (Blackman, et al., 1990, J. Exp. Med. 172, 379-382). The apparent structure of MSP-1$_{19}$ is complex, containing 12 cysteines within a span of 100 amino acid residues, and is arranged as two tandem domains that are homologous with epidermal growth factor (EGF) (Blackman, et al., 1991, supra; Morgan et al., 2000, J. Biomol. NMR 17, 337-347). Each putative EGF-domain contains six cyteine residues that would form three disulfide bridges per domain, which force the assembly of several well defined discontinous epitopes (Farley and Long, 1995, Exp. Parasitol. 80, 328-332; McBride and Heidrich, 1987, supra; Uthaipibull et al, 2001, J. Mol. Biol. 307, 1381-1394).

Because age-dependent development of immunity to malaria is due, at least in part, to antibody against erythrocytic stage parasites (Cohen, S. et al., 1964, Nature 192, 733-737), a malaria vaccine should induce effective antibodies against this developmental stage. Evidence supporting the use of MSP-1$_{42}$ and MSP-1$_{19}$ in a malaria vaccine is extensive. MSP-1$_{19}$-specific mAbs inhibit *P. falciparum* growth *in vitro* (Blackman et al., 1990, supra) and passively protect mice against infection with *P. yoelii* (Majarian et al., 1984, J. Immunol. 132, 3131-3137; Ling et al., 1994, Parasite Immunol. 16, 63-67). Immunization of *Aotus* monkeys with native *P. falciparum* MSP-1 (Siddiqui, et al.,

1987, Proc. Natl. Acad. Sci. USA 84, 3014-3018), or *S. cerevisiae* recombinant MSP-1$_{19}$ (Kumar et al., 1995, Mol. Med. 1, 325-332; Egan et al., 2000, Infect. Immun. 68, 1418-1427; Stowers et al. 2001, Trends Parasitol. 17, 415-419), protect against a homologous challenge. *E. coli*-expressed *P. yoelii* MSP-1$_{19}$ (Burns et al., 1989, J. Immunol. 143, 2670-2676) protects against a homologous challenge in rodent models. Antibodies raised against yeast MSP-1$_{19}$ grown in yeast weakly inhibit *Plasmodium* growth *in vitro* (Gozalo et al., 1998, Am. J. Trop. Med. Hyg. 59, 991-997) however this antigen lacks correct structure and induces a strong allergic response (Keitel, W. A., 1999, Vaccine 18, 531-539). MSP-1$_{19}$ may not be an optimal vaccine because it does not induce strong T-helper cell responses (Quin et al., 2001, Eur. J. Immunol. 31, 72-81). Poor MSP-1$_{19}$ T-cell immunogenicity may be a consequence of its structural stability, which allows it to resist proteolysis, and therefore to resist processing and presentation to the immune system.

Thus, MSP-1$_{42}$ may be a better choice as a vaccine candidate (Quin and Langhorne, 2001, Infect. Immun. 69, 2245-2251). Immunization of *Aotus* monkeys with baculovirus-expressed recombinant MSP-1$_{42}$, protects against a homologous challenge and the anti-sera raised, inhibit *P. falciparum* growth *in vitro*, but do not result in sufficient yield and are not yet available in clinical grade ((Chang et al., 1996, Infect. Immun. 64, 253-261; Chang et al., 1992, J. Immunol. 149, 548-555). Monoclonal antibodies that either protect against infection *in vivo* (Burns et al., 1989, J. Immunol. 143, 2670-2676), or inhibit parasite growth *in vitro* (Blackman et al., 1990, supra), are specific for discontinuous epitopes since they do not react with disulfide-reduced MSP-1$_{19}$

(McBride and Heidrich, 1987, supra; Farley and Long,
1995, Exp. Parasitol. 80, 328-332). Thus, a
recombinant vaccine produced from this part of the
MSP-1 will require correct disulfide-dependent
5   conformation to elicit a protective antibody response.
     Therefore, heterologous expression of recombinant
molecules must replicate the conformation and
structure of these proteins to induce an appropriate
immune response. Heterologous expression of
10   recombinant (MSP-1$_{42}$) from eukaryotic expression
systems, i.e. baculovirus and yeast, have lead to
recombinant molecules that possess post-translational
modifications due to N-glycosylation, and are
expressed poorly or are misfolded. Post-translational
15   modification due to N-glycosylation may be problematic
for malaria vaccines because malaria parasites lack
this ability.
     Other attempts at producing MSP-1 in *E. coli* have
not produced protective vaccines (Kumar, S. et al.,
20   1995, Molecular Medicine 1, 325-332) due to problems
with endotoxin contamination and possibly to an
inability to establish correct disulfide bridging
patterns.
     Previous attempts show that not only is a good
25   expression system needed for proper and sufficient
expression of MSP-1$_{42}$ but, in addition, a good
purification protocol is required which removes
endotoxin contamination but which retains the proper
folding of the antigen for presentation to the immune
30   system.

<div align="center">**SUMMARY OF THE INVENTION**</div>

     The present invention satisfies the needs
discussed above. The present invention provides a
method for proper expression and purification of the
35   MSP-1$_{42}$ 3D7 allele. The method of the present

invention results in elimination of contaminating proteins and conservation of the native folding and disulfide bridging of the protein. Therefore, the essentially purified MSP-1$_{42}$ protein of the present

5 invention retains proper conformation for optimal reactivity for vaccine and screening purposes.

Therefore, a major aim of the present invention resides in the production of large amounts of MSP-1$_{42}$ which maintain conformational epitopes critical to

10 epitope formation in pure form (>95% pure) for diagnostic, prophylactic and therapeutic purposes.

This may not seem complicated but, as with most strategies for protein purification, proved to be difficult and unpredictable. *E. coli* was chosen as a

15 host, even though it had gone out of favor, for two reasons: (1) *E. coli* was known to produce high level of recombinant proteins and (2) recombinant proteins produced in *E. coli* are not glycosylated, which is consistent with the capabilities of malaria parasites.

20 Several hurdles had to be overcome to achieve the desired expression level in soluble cytoplasmic form which can be sufficiently purified from host cell proteins without sacrificing proper folding of the protein. Problems with *E. coli* endotoxin levels,

25 antibiotic resistance and the presence of non-MSP-1$_{42}$ contaminants had to be resolved.

The final expression construct, pETATPfMSP-1$_{42}$(3D7) (deposited with ATCC under the Budapest Treaty on ----, accession number ----), was the product of a

30 series of subclonings, with each successive construction reducing the amount of expressed non-MSP-1 sequence. The construction of a DNA vector expressing a *P. falciparum* 3D7 MSP-1$_{42}$ molecule proceeded through several steps. A full-length fusion

35 with *E. coli* thioredoxin at the N-terminus of MSP-1$_{42}$

was prepared by cloning in the multiple cloning region of the pET32a expression vector (Construct #1, Figure 1A, pET-Trx42). The expressed protein is identified in SEQ ID NO:1. Positive clones were transformed into

5 the highly regulatable T7 RNA polymerase expressing host. Mini-induction experiments were conducted to optimize expression levels of several clones. In these experiments some variables that were investigated included induction temperature,

10 concentration of inducer (IPTG), length of time of induction, and the influence of *E. coli* host background on levels of expression [BL21(DE3) versus AD494 (DE3)]. These variables have been shown to affect the levels of expression and the partitioning

15 of protein in either soluble or insoluble fractions. SDS-PAGE and immunoblotting analysis of crude extracts from cells induced at 37°C showed that the full length fusion, trxA-MSP-1$_{42}$ (Construct #1, Figure 1A) comprised greater than 20% of the total *E. coli*

20 protein. However, following cell lysis, all of the fusion protein partitioned into the insoluble fraction and was associated with inclusion bodies. This situation is often the case with heterologous proteins that are expressed at high levels in *E. coli*.

25 Lowering the culture temperature sequentially from 37°C to 25°C during induction of expression resulted in increasing levels of soluble fusion protein in the post-sonication supernatant. By increasing the soluble protein at this stage, a urea

30 solubilization and refolding step is avoided thereby assuring more native folding of the protein. The post sonication soluble supernatant was applied to a Ni^{+2}=NTA agarose affinity column (QIAGEN) and bound protein was eluted with stepwise increasing gradients

35 of imidazole. The expressed thioredoxin-MSP-1$_{42}$ fusion

products from these cells was reactive with mAb 5.2
(see Table 1) on immunoblots. Our data suggested that
this expression system would provide sufficient levels
of recombinant protein for development as a diagnostic
5 and vaccine antigen, providing proper covalent
disulfide bridging could be achieved.

A second construct was designed (Construct #2,
pET(50)MSP-1$_{42}$) to delete the *E. coli* trxA gene
(thioredoxin protein) from Construct #1 (thioredoxin-
10 MSP-1$_{42}$ fusion). This product was developed as an
alternative to the full-length thioredoxin fusion to
address potential FDA regulatory concerns with a
thioredoxin-MSP-1$_{42}$ fusion protein vaccine. The
product formed retains the His6-tag for affinity
15 purification and an additional vector encoded sequence
(approximately 50 amino acids) which include an
enterokinase cleavage site, and S-peptide tag, and the
thrombin cleavage site fused to the N-terminus of MSP-
1$_{42}$. The expressed product is identified in SEQ ID
20 NO:2. The levels of expression from this construct
were estimated at approximately 5-10% of the total *E.
coli* protein from crude cell lysates and protein was
purified to near homogeneity (>85%) with two
consecutive passes over a Ni^{+2}-NTA agarose resin.

Table 1 - MSP-119-Specific Monoclonal Antibodies

Antibody	Immunizing parasite	Stages	Epitope/location	Ref.
2.2-7	Thai-K1	smr*	Conserved/EGF-like domain 1	McKay
12.8-2-1	Thai-T9-96 (K1 like)	smr	Conserved/EGF-like domain 1	Conway
7.5-1	Thai-K1	smr	Semi-conserved/EGF-like domain 1	McBride
12.10-5-1	Thai-T9-96 (K1 like)	smr	Semi-conserved,EGF-like domain 1&2	Blackman
5.2	Uganda-Palo Alto (3D7 like)	smr	Semi-conserved, EGF-like domain 1	Chang
7F1	Malayan Camp (3D7 like)	sm	MSP-133Strain Specific	Lyon
Polyclonal	3D7 MSP-1-42	smr	Many epitopes	Angov

*-schizonts, merozoites, rings

References:

Blackman, M.J., et. al. 1990, J. Exp. Med. 172:379-382.

Conway, D.J., et al. 1991, Parasitol. 103:1-6.

Chang, S.P. et. al. 1988, Exp. Parasitol. 67:1-11.

Mackay, M, et. al. 1985, EMBO J. 4:3823-3829.

McBride, J.S., et. al. 1982, Science, 217:254-257.

Lyon, J.A. et al. 1987, J. Immunology. 138: 895-901.

Polyclonal rabbit MSP-142 antibody was prepared at Walter Reed Army Institute of Research Department of Immunology, by immunization of rabbits with recombinant E. coli-expressed MSP-142 (3D7) adjuvanted with Complete Freunds Adjuvant.

Since the levels of expression and apparent
protein folding of Construct #2 suggested that a
correctly folded non-thioredoxin-fused MSP-1$_{42}$ was
5   expressible, a third construct was developed to remove
the entire vector non-MSP-1 encoded sequence fused at
the N-terminus of Construct #2. This upstream gene
sequence was deleted and was replaced with an annealed
oligonucleotide linker to regenerate the His6-tag
10   (Construct #3, pET42A). Therefore, Construct #3
contains 9 non-MSP-1$_{42}$ amino acids that include the
His6 and 3 linker amino acids. The expressed product
is identified in SEQ ID NO:3. The non-fused MSP-1$_{42}$
molecule from this construct is produced to adequate
15   levels (2-5%) of the total *E. coli* protein and is
correctly folded based on immunoreactivity with a
series of MSP-1$_{19}$ specific mAbs (See Table 1).
Potential regulatory concerns over selection in the
presence of ampicillin resulted in a final
20   modification on the His6-MSP-1$_{42}$ construct (Construct
#3) that included the gene for tetracycline (Construct
#4, pET-IEGR-MSP142(AT), Figure 1B). Therefore, the
plasmid designated as Construct #4 (also His6-MSP-1$_{42}$,
but selectable with tetracycline) can be selected in
25   the presence of tetracycline alone during large-scale
fermentation or with ampicillin, as necessary. The
expressed protein of Construct #4 is identified in SEQ
ID NO:3. The final plasmid pET(AT)PfMSP-1$_{42}$(3D7) was
created by removing the residual Factor Xa cleavage
30   site. Constructs 1-4 can be used to produce fusion
proteins with MSP-1$_{42}$ as a source of soluble antigen.

Intensive investigation of variables that affect
the efficiency of fermentation and induction of
expression were required to optimize His6-MSP-1$_{42}$
35   expression. Some variables which have a significant

effect on target protein yields and bear upon
purification strategies include the effects of media
composition, amount of inducer necessary, temperature
at which inducer is added to the cultures, and length

5 of time of induction, to name a few.

A low temperature of induction was necessary in
order to obtain soluble protein. The temperature of
the cultures had to be reduced from 37°C to about 25°C
prior to induction. At all higher temperatures,

10 protein was found in inclusion bodies and difficult to
isolate. Similarly, we found that the time of
induction was important for proper and maximal
expression of the protein. The length of IPTG
induction was most advantageous at 2-3 hours.

15 Induction for less time resulted in suboptimal protein
synthesis and induction for more time resulted in loss
of product due to lysis and protein degradation.

Cells were suspended in lysis buffer and lysed by
microfluidization (Microfluidics) in one pass while

20 the temperature of the sample was maintained below
10°C at all times to reduce proteolysis. The lysate
was centrifuged and the pellets and supernates were
evaluated by immunoblotting.

The clarified lysate was then purified by several

25 methods. The first method is using the ability of the
His6-tag sequence expressed as a short N-terminal
fusion on the target protein to bind to divalent
cations, i.e. nickel, immobilized onto matrices. The
w/v cell paste to resin ratio were varied to optimize

30 yield and purity of the product and to minimize cost.

After elution of the protein from the Ni^{2+} chelate
resin, it is allowed to incubate at 0-4°C overnight to
promote disulfide bridge formation. This step is
required because protein disulfie bridge formation

35 does not occur readily in the cytoplasm of the *E. coli*

BL21 DE3 expression host due to the reducing nature of
this environment. This explains our observation that
monoclonal antibodies known to react with properly
folded disulfide bridged MSP-1$_{42}$ do not react with this
5 protein either in cell lysates, or with partially
purified recombinant MSP-1$_{42}$ immediately after its
elution from the Ni^{+2} NTA affinity column. Incubating
the eluted protein at 0-4°C for up to 48 hours
following the Ni^{+2} chromatography promotes proper
10 disulfide bridge fromation because after this
incubation, all of the monoclonal antibodies now react
with the antigen. These data suggest that at the time
of lysis and chromatography on the Ni^{+2} NTA affinity
column the protein is probably folded properly through
15 ionic and hydrophobic interactions, but that the
disulfide bridges do not form until the protein is
maintained in an oxidative environment over the
observed period of time.

The eluted sample was then applied to a Q ion
20 exchange chromatography column. Binding of the sample
to the column was checked at varying pH values ranging
from pH 6.2 to 9.9. In order to define a pH condition
at which the protein would bind to the Q resin, we
varied buffer types, e.g. phosphate (pH 6-9) vs.
25 glycine (pH 8.5-11) vs. citrate (pH 4-5-6). Under the
final conditions selected, MSP-1$_{42}$ partitioned into the
flow through and most residual host cell remained
proteins bound to the column.

Finally, CM ion exchange chromatography was used
30 to remove low levels of residual *E. coli* protein and
endotoxin. Variables optimized included the binding
of protein to the column, the salt concentration of
the washing and eluting solution.

The purified *P. falciparum* MSP-1$_{42}$ was used as a
35 vaccine along with an adjuvant, for example, ADJUVANT

B, and was found to elicit malaria specific antibody responses in monkeys. More importantly, vaccination with the MSP-1$_{42}$ elicited neutralizing antibodies and protects monkeys against a malaria infection.

5   Therefore, it is an object of the present invention to provide a recombinant *P. falciparum* MSP-1$_{42}$ for use in diagnostic assays and for production of antibodies.

    It is another object of the present invention to 10 provide compositions comprising purified recombinant *P. falciparum* MSP-1$_{42}$.

    It is yet another object of the present invention to provide novel vector constructs for recombinantly expressing *P. falciparum* MSP-1$_{42}$, as well as host cells 15 transformed with said vector.

    It is also an object of the present invention to provide a method for producing and purifying recombinant *P. falciparum* MSP-1$_{42}$ protein comprising:

    growing a host cell containing a vector 20 expressing *P. falciparum* MSP-1$_{42}$ proteins in a suitable culture medium,

    causing expression of said vector sequence as defined above under suitable conditions for production of soluble protein and,

25   lysing said transformed host cells and recovering said MSP-1$_{42}$ protein such that it retains its native folding and is essentially free of host toxins.

    It is also an object of the present invention to provide diagnostic and immunogenic uses of the 30 recombinant *P. falciparum* MSP-1$_{42}$ protein of the present invention, as well as to provide kits for diagnostic use for example in malaria screening and confirmatory antibody tests.

    It is also an object of the present invention to 35 provide monoclonal or polyclonal antibodies, more

particularly human monoclonal antibodies or mouse monoclonal antibodies which are humanized, which react specifically with MSP-1$_{42}$ epitopes, either comprised in peptides or conformational epitopes comprised in
5   recombinant proteins.

It is also an object of the present invention to provide possible uses of anti-MSP-1$_{42}$ monoclonal antibodies for malaria antigen detection or for therapy of chronic malaria infection.
10   It is yet another object of the present invention to provide a malaria vaccine comprising MSP-1$_{42}$.of the present invention, in an amount effective to elicit an immune response in an animal against *P. falciparum*; and a pharmaceutically acceptable
15   diluent, carrier, or excipient.

It is another object of the present invention to provide a method for eliciting in a subject an immune response against malaria, the method comprising administering to a subject a composition comprising
20   MSP-1$_{42}$ of the present invention. In one aspect of the invention, the DNA vaccine is delivered along with an adjuvant, for example ADJUVANT B.

It is another object of the present invention to provide a method for preventing malaria infection in
25   an animal comprising administering to the animal the MSP-1$_{42}$ of the present invention.

The vaccine according to the present invention is inherently safe, is not painful to administer, and should not result in adverse side effects to the
30   vaccinated individual.

The present invention also provides vectors for the production of a recombinant MSP-1$_{42}$, host cells containing the vectors, a method for fermenting and inducing the host cells, and a method for isolating
35   and purifying the recombinant protein. Also provided

is a method for bulk fermentation and expression of MSP-1$_{42}$.

All the objects of the present invention are considered to have been met by the embodiments as set out below.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A: pET32a plasmid map.

Figure 1B: pET(AT)PfMSP-1$_{42}$ plasmid map.

Figure 2A, 2B and 2C: SDS-PAGE analysis of MSP-1$_{42}$ during Purification and of the Final Product (FMP1). Protein detected by Coomassie Blue staining (2A and 2B) and immunoblotting (2C). 2A, non-reduced samples: lane 1, nickel chelate eluate; lane 2, Q flow through; lane 3 CM eluate. 2B, 10 ug FMP1 electrophoresed under non-reduced (lane 1) or reduced (lane 2) conditions. 2C, immunoblotting of 1 ug of FMP electrophoresed under non-reducing: lane 1 mAb 7F1; lane 2, mAb 12.10; lane 3, rabbit anti-*E. coli* antiserum.

Figure 3: FMP1 Stability. Commassie Blue stained non-reduced SDS-PAGE gel with 10 ug samples of FMP1 stored for 18 months under various conditions. Lane 1, -80°C; lane 2, -20°C; lane 3, 4°C.

Figure 4: FMP1 Immunogenicity. Rhesus monkeys were boosted 1, 3, 5, and 7 months after priming with FMP1/ADJUVANT B (rectangles) or FMP1/alum (circles). Sera were collected just prior to immunization (arrows) and two weeks after each immunization. In the case of the ADJUVANT B cohort serum collection continued monthly for 9 months. Antibody titers were measured by IFA (filled symbols) and kinetic ELISA (open symbols).

Figure 5: Merozoite Invasion Inhibition. Triplicate synchronous cultures of *P. falciparum* schizont-infected erythrocytes were incubated for 24 hours with the IgG fractions of pre-immune (open

diamonds) or immune (filled diamonds) IgG fractions from rabbits immunized with FMP1 in Fruends adjuvents. IgG was tested at doses of 200, 400, and 800 ug/ml and parasite growth was quantified by counting 5000 erythroctyes or 100 parasitized erythrocytes, whichever occurred first. Data are shown as the mean parasitemia and 95% confidence interval.

Figure 6: CMI responses to FMP1/ADJUVANT B. Peripheral blood mononuclear cells (PBMC) were stimulated with FMP1 (top) or *P. falciparum* parasitized erythrocytes (PBRC, bottom) and proliferation was measured by 3H-thymidine uptake. Each panel contains data from all individuals within a vaccine group and individuals are differentiated by symbol. Samples were collected prior to immunization (pre) and two weeks following each immunization (post 1, post 2, post 3).

DETAILED DESCRIPTION

In the description that follows, a number of terms used in recombinant DNA, parasitology and immunology are extensively utilized. In order to provide a clearer and consistent understanding of the specification and claims, including the scope to be given such terms, the following definitions are provided.

In general, an 'epitope' is defined as a linear array of 3-10 amino acids aligned along the surface of a protein. In a linear epitope, the amino acids are joined sequentially and follow the primary structure of the protein. In a conformational epitope, residues are not joined sequentially, but lie linearly along the surface due to the conformation (folding) of the protein. With respect to conformational epitopes, the length of the epitope-defining sequence can be subject to wide variations. The portions of the primer

structure of the antigen between the residues defining
the epitope may not be critical to the structure of
the conformational epitope. For example, deletion or
substitution of these intervening sequences may not
5   affect the conformational epitope provided sequences
critical to epitope conformation are maintained (e.g.
cysteines involved in disulfide bonding, glycosylation
sites, etc.). A conformational epitope may also be
formed by 2 or more essential regions of subunits of a
10 homo-oligomer or hetero-oligomer. As used herein,
`epitope` or `antigenic determinant` means an amino
acid sequence that is immunoreactive. As used herein,
an epitope of a designated polypeptide denotes
epitopes with the same amino acid sequence as the
15 epitope in the designated polypeptide, and immunologic
equivalents thereof. Such equivalents also include
strain, subtype (=genotype), or type(group)-specific
variants, e.g. of the currently known sequences or
strains belonging to *Plasmodium* such as 3D7, FVO and
20 CAMP, or any other known or newly defined *Plasmodium*
strain.

The term `solid phase` intends a solid body to
which the individual *P. falciparum* antigen is bound
covalently or by noncovalent means such as
25 hydrophobic, ionic, or van der Waals association.

The term `biological sample` intends a fluid or
tissue of a mammalian individual (e.g. an anthropoid,
a human), reptilian, avian, or any other zoo or farm
animal that commonly contains antibodies produced by
30 the individual, more particularly antibodies against
malaria. The fluid or tissue may also contain *P.
falciparum* antigen. Such components are known in the
art and include, without limitation, blood, plasma,
serum, urine, spinal fluid, lymph fluid, secretions of
35 the respiratory, intestinal or genitourinary tracts,

tears, saliva, milk, white blood cells and myelomas.
Body components include biological liquids. The term
`biological fluid` refers to a fluid obtained from an
organism. Some biological fluids are used as a source

5 of other products, such as clotting factors (e.g.
Factor VlI1;C), serum albumin, growth hormone and the
like.

The term `immunologically reactive` means that
the antigen in question will react specifically with

10 anti-MSP-1 antibodies present in a body component from
a malaria infected individual.

The term `immune complex` intends the combination
formed when an antibody binds to an epitope on an
antigen.

15 The term `MSP-1$_{42}$' as used herein refers to the
polymorphic C-terminal 42 kDa protein fragment or
polypeptide resulting from the processing by proteases
of the 195 kDa membrane-anchored MSP-1 precursor.
During merozoite invasion, the 42 kDa fragment is

20 subjected to secondary processing producing a 33-kDa
fragment (MSP-1$_{33}$) and a 19 kDa C-terminal fragment,
(MSP-1$_{19}$) which remains attached via GPI to the surface
of the invading merozoite. The MSP-1$_{42}$ protein extends
from approximately amino acid (aa) 1327 to about aa

25 1700 of the full-length precursor protein (Genbank
accession #Z35327).

The term 'MSP-1$_{42}$' as used herein also includes
analogs and truncated forms that are immunologically
cross-reactive with natural MSP-1$_{42}$. By 'MSP-1$_{42}$' is

30 intented MSP-1$_{42}$ from other strains of *Plasmodium*, or
any other newly identified strain of *Plasmodium*.

The term `homo-oligomer` as used herein refers to
a complex of MSP-1$_{42}$ containing more than one MSP-1$_{42}$
monomer, e.g. MSP-1$_{42}$/MSP-1$_{42}$ dimers, trimers or

tetramers, or any higher-order homo-oligomers of MSP-1_{42} are all `homo-oligomers` within the scope of this definition. The oligomers may contain one,
two, or several different monomers of MSP-1_{42} obtained

5   from different strains of *Plasmodium falciparum* including for example 3D7, Camp, FVO, and others. Such mixed oligomers are still homo-oligomers within the scope of this invention, and may allow more universal diagnosis, prophylaxis or treatment of malaria.

10   The term `purified` as applied to proteins herein refers to a composition wherein the desired protein comprises at least 35% of the total protein component in the composition. The desired protein preferably comprises at least 40%, more preferably at

15   least about 50%, more preferably at least about 60%, still more preferably at least about 70%, even more preferably at least about 80%, even more preferably at least about 90%, and most preferably at least about 95% of the total protein component. The composition

20   may contain other compounds such as carbohydrates, salts, lipids, solvents, and the like, without affecting the determination of the percentage purity as used herein. An `isolated` MSP-1_{42} protein intends a *Plasmodium* protein composition that is at least 35%

25   pure.
       The term `essentially purified proteins` refers to proteins purified such that they can be used for *in vitro* diagnostic methods and as a prophylactic compound. These proteins are substantially free from

30   cellular proteins, vector-derived proteins or other *Plasmodium* components. The proteins of the present invention are purified to homogeneity, at least 80% pure, preferably, 90%, more preferably 95%, more preferably 97%, more preferably 98%, more preferably

35   99%, even more preferably 99.5%.

The term `recombinantly expressed` used within the context of the present invention refers to the fact that the proteins of the present invention are produced by recombinant expression methods be it in
5   prokaryotes, or lower or higher eukaryotes as discussed in detail below.

The term `lower eukaryote` refers to host cells such as yeast, fungi and the like. Lower eukaryotes are generally (but not necessarily) unicellular.
10   Preferred lower eukaryotes are yeasts, particularly species within *Saccharomyces*. *Schizosaccharomyces*, *Kluveromyces*, *Pichia* (e.g. *Pichia pastoris*), *Hansenula* (e.g. *Hansenula polymorpha*, *Yarowia*, *Schwaniomyces*, *Schizosaccharomyces*, *Zygosaccharomyces* and the like.
15   *Saccharomyces cerevisiae*, *S. carlsberoensis* and *K. lactis* are the most commonly used yeast hosts, and are convenient fungal hosts.

The term `prokaryotes` refers to hosts such as *E.coli*, *Lactobacillus*, *Lactococcus*, *Salmonella*,
20   *Streptococcus*, *Bacillus subtilis* or *Streptomyces*. Also these hosts are contemplated within the present invention.

The term `higher eukaryote` refers to host cells derived from higher animals, such as mammals,
25   reptiles, insects, and the like. Presently preferred higher eukaryote host cells are derived from Chinese hamster (e.g. CHO), monkey (e.g. COS and Vero cells), baby hamster kidney (BHK), pig kidney (PK15), rabbit kidney 13 cells (RK13), the human osteosarcoma cell
30   line 143 B, the human cell line HeLa and human hepatoma cell lines like Hep G2, and insect cell lines (e.g. Spodoptera frugiperda). The host cells may be provided in suspension or flask cultures, tissue cultures, organ cultures and the like. Alternatively
35   the host cells may also be transgenic animals.

The term `polypeptide` refers to a polymer of amino acids and does not refer to a specific length of the product; thus, peptides, oligopeptides, and proteins are included within the definition of

5   polypeptide. This term also does not refer to or exclude post-expression modifications of the polypeptide, for example, glycosylations, acetylations, phosphorylations and the like. Included within the definition are, for example, polypeptides

10 containing one or more analogues of an amino acid (including, for example, unnatural amino acids, PNA, etc.), polypeptides with substituted linkages, as well as other modifications known in the art, both naturally occurring and non-naturally occurring.

15   The term `recombinant polynucleotide or nucleic acid` intends a polynucleotide or nucleic acid of genomic, cDNA, semisynthetic, or synthetic origin which, by virtue of its origin or manipulation : (1) is not associated with all or a portion of a

20 polynucleotide with which it is associated in nature, (2) is linked to a polynucleotide other than that to which it is linked in nature, or (3) does not occur in nature.

        The term `recombinant host cells`, `host cells`,

25 `cells`, `cell lines`, `cell cultures`, and other such terms denoting microorganisms or higher eukaryotic cell lines cultured as unicellular entities refer to cells which can be or have been, used as recipients for a recombinant vector or other transfer

30 polynucleotide, and include the progeny of the original cell which has been transfected. It is understood that the progeny of a single parental cell may not necessarily be completely identical in morphology or in genomic or total DNA complement as

the original parent, due to natural, accidental, or deliberate mutation.

The term `replicon` is any genetic element, e.g., a plasmid, a chromosome, a virus, a cosmid, etc., that behaves as an autonomous unit of polynucleotide replication within a cell; i.e., capable of replication under its own control.

The term `vector` is a replicon further comprising sequences providing replication and/or expression of a desired open reading frame.

The term `control sequence` refers to polynucleotide sequences which are necessary to effect the expression of coding sequences to which they are ligated. The nature of such control sequences differs depending upon the host organism; in prokaryotes, such control sequences generally include promoter, ribosomal binding site, and terminators; in eukaryotes, generally, such control sequences include promoters, terminators and, in some instances, enhancers. The term `control sequences` is intended to include, at a minimum, all components whose presence is necessary for expression, and may also include additional components whose presence is advantageous, for example, leader sequences which govern secretion.

The term `promoter` is a nucleotide sequence which is comprised of consensus sequences which allow the binding of RNA polymerase to the DNA template in a manner such that mRNA production initiates at the normal transcription initiation site for the adjacent structural gene.

The expression `operably linked` refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. A control sequence `operably linked` to a coding sequence is ligated in such a way that

expression of the coding sequence is achieved under conditions compatible with the control sequences.

An `open reading frame` (ORF) is a region of a polynucleotide sequence which encodes a polypeptide

5   and does not contain stop codons; this region may represent a portion of a coding sequence or a total coding sequence.

A `coding sequence` is a polynucleotide sequence which is transcribed into mRNA and/or translated into

10   a polypeptide when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a translation start codon at the 5'-terminus and a translation stop codon at the 3'-terminus. A coding sequence can

15   include but is not limited to mRNA, DNA (including cDNA), and recombinant polynucleotide sequences.

The term `immunogenic` refers to the ability of a substance to cause a humoral and/or cellular response, whether alone or when linked to a carrier, in the

20   presence or absence of an adjuvant. `Neutralization` refers to an immune response that blocks the infectivity, either partially or fully, of an infectious agent. A `vaccine` is an immunogenic composition capable of eliciting protection against

25   malaria, whether partial or complete. A vaccine may also be useful for treatment of an infected individual, in which case it is called a therapeutic vaccine.

The term `therapeutic` refers to a composition

30   capable of treating malaria infection.
The term `effective amount` for a therapeutic or prophylactic treatment refers to an amount of epitope-bearing polypeptide sufficient to induce an immunogenic response in the individual to which it is

35   administered, or to otherwise detectably immunoreact

in its intended system (e.g., immunoassay).
Preferably, the effective amount is sufficient to
effect treatment, as defined above. The exact amount
necessary will vary according to the application. For
5 vaccine applications or for the generation of
polyclonal antiserum/antibodies, for example, the
effective amount may vary depending on the species,
age, and general condition of the individual, the
severity of the condition being treated, the
10 particular polypeptide selected and its mode of
administration, etc. It is also believed that
effective amounts will be found within a relatively
large, non-critical range. An appropriate effective
amount can be readily determined using only routine
15 experimentation. Preferred ranges of MSP-1$_{42}$ for
prophylaxis of malaria disease are about 0.01 to 1000
ug/dose, more preferably about 0.1 to 100 ug/dose,
most preferably about 10-50 ug/dose. Several doses may
be needed per individual in order to achieve a
20 sufficient immune response and subsequent protection
against malaria.

More particularly, the present invention
contemplates essentially purified MSP-1$_{42}$ and a method
25 for isolating or purifying recombinant MSP-1$_{42}$
protein, characterized in that upon lysing the
transformed host cells to isolate the recombinantly
expressed protein, the disulfide bonds necessary for
proper folding of the protein are preserved.
30 The term `MSP-1$_{42}$' refers to a polypeptide or an
analogue thereof (e.g. mimotopes) comprising an amino
acid sequence (and/or amino acid analogues) defining
at least one MSP-1$_{42}$ epitope. Typically, the sequences
defining the epitope correspond to the amino acid
35 sequence of MSP-1$_{42}$ region of *P. falciparum* (either

identically or via substitution of analogues of the
native amino acid residue that do not destroy the
epitope). The MSP-1$_{42}$ protein corresponds to a
nucleotide sequence identified in SEQ ID NO:5 and

5   spans from amino acid 1327 to 1701 of MSP-1 3D7 allele
(SEQ ID NO:6). Upon expression in the parasite system
(non-glycosylated), it is believed to have an
approximate molecular weight of 42 kDa as determined
by SDS-PAGE. It is understood that these protein

10 endpoints are approximations (e.g. the carboxy
terminal end of MSP-1$_{42}$ could lie somewhere in the 1700
to 1720 amino acid region). The absolute C-terminus
is not defined due to the post-translational
modification that transfers MSP-1 to a GPI lipid

15 membrane anchor.

The MSP-1$_{42}$ antigen used in the present invention
is preferably a full-length protein, or a
substantially full-length version, i.e. containing
functional fragments thereof (e.g. fragments which are

20 not missing sequence essential to the formation or
retention of an epitope). Furthermore, the *P.
falciparum* antigen of the present invention can also
include other sequences that do not block or prevent
the formation of the conformational epitope of

25 interest. The presence or absence of a conformational
epitope can be readily determined though screening the
antigen of interest with an antibody as described in
the Examples below (polyclonal serum or monoclonal to
the conformational epitope) and comparing its

30 reactivity to that of a denatured version of the
antigen which retains only linear epitopes (if any).

The *P. falciparum* antigen of the present
invention can be made by any recombinant method that
provides the epitope of interest. For example,

35 recombinant expression in *E. coli* is a preferred

method to provide non-glycosylated antigens in
`native` conformation. This is most desirable because
natural *P. falciparum* antigens are not glycosylated.
Proteins secreted from mammalian cells may contain
5   modifications including galactose or sialic acids
which may be undesirable for certain diagnostic or
vaccine applications. However, it may also be possible
and sufficient for certain applications, as it is
known for proteins, to express the antigen in other
10   recombinant hosts such as baculovirus and yeast or
higher eukaryotes, as long as glycosylation is
inhibited.

The proteins according to the present invention
may be secreted or expressed within compartments of
15   the cell. Preferably, however, the proteins of the
present invention are expressed within the cell and
are released upon lysing the cells.

It is also understood that the isolates used in
the examples section of the present invention were not
20   intended to limit the scope of the invention and that
an equivalent sequence from a *P. falciparum* isolate
from another allele, e.g. FVO, or CAMP, can be used to
produce a recombinant MSP-1_{42} protein using the methods
described in the present application. Other new
25   strains of *Plasmodium* may be a suitable source of MSP-
1_{42} sequence for the practice of the present invention.

The MSP-1_{42} protein of the present invention is
expressed as part of a recombinant vector. The
present invention relates more particularly to the
30   MSP-1_{42} nucleic acid sequence in recombinant nucleic
acids pETATpfMSP-1_{42}(3D7) as represented in SEQ ID NO:7
or parts thereof. The MSP-1_{42} genomic sequence was
cloned into pET32a from Novagen (Madison, Wisconsin).
This plasmid comprises, in sequence, a T7 promoter,
35   optionally a lac operator, a ribosome binding site,

restriction sites to allow insertion of the structural gene and a T7 terminator sequence. Other vectors provided include pET-Trx42, pET(50)MSP-1$_{42}$, pET42A, pET-IEGR-MSP-1$_{42}$(AT) all described below in Materials

5   and Methods. Examples of other plasmids which contain the T7 inducible promoter include the expression plasmids pET-17b, pET-11a, pET-24a-d(+), and pEt-9a, all from Novagen (Madison, Wisconsin); see the Novagen catalogue.

10   The present invention also contemplates host cells transformed with a recombinant vector as defined above. In a preferred embodiment, *E. coli* strain BL21 (DE3) (F-ompT hsdSB(rB-mB-) gal dcm (DE3)) is employed. The above plasmids may be transformed into

15   this strain or other strains of *E. coli* having the following characteristics: a T7 RNA polymerase rec gene, Lon, ompT protease mutants or any other *E. coli* with a protease deficiency such as *E. coli* origami. Preferably, the host includes BL21(DE3) and any of its

20   precursors. Other host cells such as insect cells can be used taking into account that other cells may result in lower levels of expression.

Eukaryotic hosts include lower and higher eukaryotic hosts as described in the definitions

25   section. Lower eukaryotic hosts include yeast cells well known in the art. Higher eukaryotic hosts mainly include mammalian cell lines known in the art and include many immortalized cell lines available from the ATCC, inluding HeLa cells, Chinese hamster ovary

30   (CHO) cells, Baby hamster kidney (BHK) cells, PK15, RK13 and a number of other cell lines. MSP-1$_{42}$ expressed in these cells will be glycosylated unless the cells have been altered such that glycosylation of the recombinant protein is not possible. It is

35   expected that when producing MSP-1$_{42}$ in a eukaryotic

expression system, extensive investigation into
methods for expressing, isolating, purifying, and
characterizing the protein would be required as
eukaryotic cells post-translationally modify this
5   protein and this would alter protein structure and
immunogenicity.

Methods for introducing vectors into cells are
known in the art.   Please see e.g., Maniatis, Fitsch
and Sambrook, <u>Molecular Cloning; A Laboratory</u> Manual
10   (1982) or <u>DNA Cloning</u>, Volumes I and II (D. N. Glover
ed. 1985) for general cloning methods.   Host cells
provided by this invention include *E. coli* containing
pET-Trx42, *E. coli* containing pET(50)MSP-1$_{42}$,, and *E.
coli* containing pET42A, *E. coli* containing pET-IEGR-
15   MSP-1$_{42}$(AT), and *E. coli* containing pET(AT)PfMSP-
1$_{42}$(3D7).

A preferred method for isolating or purifying
MSP-1$_{42}$ as defined above is further characterized as
comprising at least the following steps:
20   (i) growing a host cell as defined above
transformed with a recombinant vector expressing MSP-
1$_{42}$ proteins in a suitable culture medium,

(ii) causing expression of said vector sequence
as defined above under suitable conditions for
25   production of a soluble protein,

(iii) lysing said transformed host cells and
recovering said MSP-1$_{42}$ protein such that it retains
its native conformation and is essentially pure.

Once the host has been transformed with the
30   vector, the transformed cells are grown in culture in
the presence of the desired antibiotic.   For FDA
regualtory purposes, it is preferable to use
tetracycline or kanamycin.   When cells reach optimal
biomass density, in this case about 0.4 OD 600 in

small culture flasks or 4-6 OD 600 in bulk fermentors, the cells are induced to produce the recombinant protein. The inventors have found after trial and error that for expression of a soluble MSP-1$_{42}$, it was

5 necessary to cool the culture to a range of about 10°C - 20°C, more preferably about 15°C - 28°C, most preferably about 24 to 26°C prior to induction. The concentration of inducer, i.e. IPTG, added affects the maximal protein synthesis. It was found that a

10 concentration of 0.1 mM IPTG was best, however, a range of 0.05 to 0.5 mM would be sufficient to produce 80-100% of maximal.

The cells were then collected and lysed to release the recombinant protein. Preferably, lysis

15 should occur at a paste to buffer ratio of 1:3 w/v to reduce viscosity and volume of sample loaded on Ni-NTA column. Preferably, lysis is in the presence of imidazole which reduces non specific binding of *E. coli* protein to Ni resin, and benzonase which is able

20 to digest *E. coli* nucleic acids at a reduced temperature. Lysis is preferably at a temperature of about 0°C - 24°C, more preferably about 5-15°C in order to retain native folding of the MSP-1$_{42}$ protein and to reduce proteolysis. A high salt concentration

25 of about 0.5-1.0 M is preferable. Salts used include NaCl or other monovalent ions.

Preferably, the *E. coli* endotoxin is separated and removed from the recombinant protein. This can be done several ways. For MSP-1$_{42}$, endotoxin was removed

30 by applying to a Ni^{+2}-NTA column. The removal of endotoxin depended on washing at low pH, about 5.8 to 6.5, preferably about pH 6.2, in high salt, about 0.5 to about 1.0 mM, preferably about 500 mM NaCl at a flow rate of about 20 to about 35 ml/min, preferably

35 about 30 ml/min. The cell paste to resin ratio can be

about 5:1 to about 7:1 w/v, preferably about 6:1 w/v.
The recombinant protein can be eluted by addition of
high pH buffer of about 7.5 to about 8.5, preferably
about pH 8.0, in a phosphate buffer of about 10-20 mM,

5   more preferably about 10 mM sodium phosphate buffer.

At this point the recombinant protein is about
50% pure. If further purity is required, ion-exchange
chromatography can be utilized. The column is
preferably with an ionic character such that a pH to

10   reduce protein binding and promote endotoxin and
nucleic acid binding can be used.

Finally, the flow through sample (about 0.1
mg/ml), can be subjected to further ion exchange
chromatography for further concentration and

15   purification. The MSP-1$_{42}$ of the present invention was
subjected to CM ion exchange chromatography. The pH
of the buffer can be about 5.2 to about 6.2,
preferably about 6.0. The salt concentration is about
25 mM to about 50 mM, preferably about 35 mM.

20   The bulk process for the isolation of purified
MSP-1$_{42}$ differs little from the process described
above. The concentration of imidazole is changed to
about 120 mM to about 200 mM, preferably about 160 mM
in order to specifically elute MSP-1$_{42}$.

25   The present invention further relates to a
composition comprising at least one of the following
MSP-1$_{42}$ peptides as listed in Table 3:
MSP-1$_{42}$ alone (SEQ ID NO:5) spanning amino acids
to 1326-1701 of MSP-1,

30   MSP-1$_{42}$ with thioredoxin from vector pET-Trx 42
(SEQ ID NO:1);
MSP-1$_{42}$ without thioredoxin from vector pET(5)MSP-
1$_{42}$ (SEQ ID NO:2);
MSP-1$_{42}$ plus His6tag produced from vector pET42A

35   (SEQ ID NO:3)

MSP-1$_{42}$ without Factor Xa from vector pET-IEGR-MSP-1$_{42}$ (AT) (SEQ ID NO:3);

MSP-1$_{42}$ plus 17 amino acids at N-terminal in final construct (SEQ ID NO :3), from construct pET(AT)PfMSP-1$_{42}$(3D7), the final expressed product referred to as FMP-1 (SEQ ID NO:7).

The present invention also relates to a composition comprising peptides or polypeptides as described above, for *in vitro* detection of malaria antibodies present in a biological sample.

The present invention also relates to a composition comprising at least one of the following MSP-1$_{42}$ conformational epitopes:

epitope recognized by monoclonal antibodies 12.10, 12.8, 7.5, 2.2, 1E1 (Blackman et al., 1990, supra; Conway et al., 1991, Parasitology 103,1-6; McBride et al., 1982, Science 217, 254-257; Mackay et al., 1985, Embo J. 4, 3823-3829).

epitope recognized by monoclonal antibody 5.2 (Chang et al., 1988, Exp. Parasitol. 67, 1-11), and

epitope recogized by monoclonal antibody 7F1 (Lyon et al., 1987, J. Immunol. 138, 895-901).

The present invention also relates to an MSP-1$_{42}$ specific antibody raised upon immunizing an animal with a peptide or protein composition, with said antibody being specifically reactive with any of the polypeptides or peptides as defined above, and with said antibody being preferably a monoclonal antibody.

The present invention also relates to an MSP-1$_{42}$ specific antibody screened from a variable chain library in plasmids or phages or from a population of human B-cells by means of a process known in the art, with said antibody being reactive with any of the polypeptides or peptides as defined above, and with said antibody being preferably a monoclonal antibody.

The MSP-1$_{42}$ specific monoclonal antibodies of the invention can be produced by any hybridoma liable to be formed according to classical methods from splenic or lymph node cells of an animal, particularly from a

5 mouse or rat, immunized against the *Plasmodium* polypeptides or peptides according to the invention, as defined above on the one hand, and of cells of a myeloma cell line on the other hand, and to be selected by the ability of the hybridoma to produce

10 the monoclonal antibodies recognizing the polypeptides which has been initially used for the immunization of the animals.

The antibodies involved in the invention can be labelled by an appropriate label of the enzymatic,

15 fluorescent, or radioactive type.

The monoclonal antibodies according to this preferred embodiment of the invention may be humanized versions of mouse monoclonal antibodies made by means of recombinant DNA technology, departing from parts of

20 mouse and/or human genomic DNA sequences coding for H and L chains from cDNA or genomic clones coding for H and L chains.

Alternatively the monoclonal antibodies according to this preferred embodiment of the invention may be

25 human monoclonal antibodies. These antibodies according to the present embodiment of the invention can also be derived from human peripheral blood lymphocytes of patients infected with malaria, or vaccinated against malaria. Such human monoclonal

30 antibodies are prepared, for instance, by means of human peripheral blood lymphocytes (PBL) repopulation of severe combined immune deficiency (SCID) mice, or by means of transgenic mice in which human immunoglobulin genes have been used to replace the

35 mouse genes.

The invention also relates to the use of the proteins or peptides of the invention, for the selection of recombinant antibodies by the process of repertoire cloning.

5 Antibodies directed to peptides or single or specific proteins derived from a certain strain may be used as a medicament, more particularly for incorporation into an immunoassay for the detection of *Plasmodium* strains for detecting the presence of MSP-

10 1_{42} antigens, or antigens containing MSP-1_{42} epitopes, for prognosing/monitoring of malaria disease, or as therapeutic agents.

Alternatively, the present invention also relates to the use of any of the above-specified MSP-1_{42}

15 monoclonal antibodies for the preparation of an immunoassay kit for detecting the presence of MSP-1_{42} antigen or antigens containing MSP-1_{42} epitopes in a biological sample, for the preparation of a kit for prognosing/monitoring of malaria disease or for the

20 preparation of a malaria medicament.

The present invention also relates to a method for *in vitro* diagnosis or detection of malaria antigen present in a biological sample, comprising at least the following steps:

25 (i) contacting said biological sample with any of the MSP-1_{42} specific monoclonal antibodies as defined above, preferably in an immobilized form under appropriate conditions which allow the formation of an immune complex,

30 (ii) removing unbound components,

(iii) incubating the immune complexes formed with heterologous antibodies, which specifically bind to the antibodies present in the sample to be analyzed, with said heterologous antibodies conjugated to a

35 detectable label under appropriate conditions,

(iv) detecting the presence of said immune complexes visually or mechanically (e.g. by means of densitometry, fluorimetry, colorimetry).

The present invention also relates to a kit for *in vitro* diagnosis of a malaria antigen present in a biological sample, comprising:

at least one monoclonal antibody as defined above, with said antibody being preferentially immobilized on a solid substrate,

a buffer or components necessary for producing the buffer enabling binding reaction between these antibodies and the malaria antigens present in the biological sample, and

a means for detecting the immune complexes formed in the preceding binding reaction.

The kit can possibly also include an automated scanning and interpretation device for inferring the malaria antigens present in the sample from the observed binding pattern.

Monoclonal antibodies according to the present invention are suitable both as therapeutic and prophylactic agents for treating or preventing malaria infection in susceptible malaria-infected subjects. Subjects include rodents such as mice or guinea pigs, monkeys, and other mammals, including humans.

In general, this will comprise administering a therapeutically or prophylactically effective amount of one or more monoclonal antibodies of the present invention to a susceptible subject or one exhibiting malaria infection. Any active form of the antibody can be administered, including Fab and $F(ab')_2$ fragments. Antibodies of the present invention can be produced in any system, including insect cells, baculovirus expression systems, chickens, rabbits, goats, cows, or plants such as tomato, potato, banana

or strawberry. Methods for the production of
antibodies in these systems are known to a person with
ordinary skill in the art. Preferably, the antibodies
used are compatible with the recipient species such
5   that the immune response to the MAbs does not result
in clearance of the MAbs before parasite can be
controlled, and the induced immune response to the
MAbs in the subject does not induce "serum sickness"
in the subject. Preferably, the MAbs administered
10 exhibit some secondary functions such as binding to Fc
receptors of the subject.

Treatment of individuals having malaria infection
may comprise the administration of a therapeutically
effective amount of MSP-1$_{42}$ antibodies of the present
15 invention. The antibodies can be provided in a kit as
described below. The antibodies can be used or
administered as a mixture, for example in equal
amounts, or individually, provided in sequence, or
administered all at once. In providing a patient with
20 antibodies, or fragments thereof, capable of binding
to MSP-1$_{42}$, or an antibody capable of protecting
against malaria in a recipient patient, the dosage of
administered agent will vary depending upon such
factors as the patient's age, weight, height, sex,
25 general medical condition, previous medical history,
etc.

In general, it is desirable to provide the
recipient with a dosage of antibody which is in the
range of from about 1 pg/kg-100 pg/kg, 100 pg/kg-500
30 pg/kg, 500 pg/kg-1 ng/kg, 1 ng/kg-100 ng/kg, 100
ng/kg-500 ng/kg, 500 ng/kg- 1 ug/kg, 1 ug/kg- 100
ug/kg, 100 ug/kg-500 ug/kg, 500 ug/kg- 1 mg/kg, 1
mg/kg-50 mg/kg, 50 mg/kg-100 mg/kg, 100 mg/kg-500
mg/kg, 500 mg/kg-1 g/kg, 1 g/kg-5 g/kg, 5 g/kg-10 g/kg

(body weight of recipient), although a lower or higher dosage may be administered.

In a similar approach, another prophylactic use of the monoclonal antibodies of the present invention
5 is the active immunization of a patient using an anti-idiotypic antibody raised against one of the present monoclonal antibodies. Immunization with an anti-idiotype which mimics the structure of the epitope could elicit an active anti-MSP-1_{42} response
10 (Linthicum, D.S. and Farid, N.R., Anti-Idiotypes, Receptors, and Molecular Mimicry (1988), pp 1-5 and 285-300).

Likewise, active immunization can be induced by administering one or more antigenic and/or immunogenic
15 epitopes as a component of a subunit vaccine. Vaccination could be performed orally or parenterally in amounts sufficient to enable the recipient to generate protective antibodies against this biologically functional region, prophylactically or
20 therapeutically. The host can be actively immunized with the antigenic/immunogenic peptide in pure form, a fragment of the peptide, or a modified form of the peptide. One or more amino acids, not corresponding to the original protein sequence can be added to the
25 amino or carboxyl terminus of the original peptide, or truncated form of peptide. Such extra amino acids are useful for coupling the peptide to another peptide, to a large carrier protein, or to a support. Amino acids that are useful for these purposes include: tyrosine,
30 lysine, glutamic acid, aspartic acid, cyteine and derivatives thereof. Alternative protein modification techniques may be used e.g., NH_2-acetylation or COOH-terminal amidation, to provide additional means for coupling or fusing the peptide to another protein or
35 peptide molecule or to a support.

The antibodies capable of protecting against
malaria are intended to be provided to recipient
subjects in an amount sufficient to effect a reduction
in the malaria infection symptoms. An amount is said

5   to be sufficient to "effect" the reduction of
infection symptoms if the dosage, route of
administration, etc. of the agent are sufficient to
influence such a response. Responses to antibody
administration can be measured by analysis of

10   subject's vital signs.

       The present invention more particularly relates
to a composition comprising at least one of the above-
specified peptides or a recombinant $MSP-1_{42}$ protein
composition as defined above, for use as a vaccine for

15   immunizing a mammal, preferably humans, against
malaria, comprising administering a sufficient amount
of the composition possibly accompanied by
pharmaceutically acceptable adjuvant(s), to produce an
immune response.

20   Immunogenic compositions can be prepared
according to methods known in the art. The present
compositions comprise an immunogenic amount of a
recombinant $MSP-1_{42}$ proteins or peptides as defined
above, usually combined with a pharmaceutically

25   acceptable carrier, preferably further comprising an
adjuvant.

       The proteins of the present invention, preferably
purified $MSP-1_{42}$ derived from $pETATPfMBP-1_{42}$ (3D7) or
FMP-1, are expected to provide a particularly useful

30   vaccine antigen, since the antigen is able to induce
invasion inhibitory antibodies as well as high titer
antibodies that react with schizont-infected
erythrocytes.

       Pharmaceutically acceptable carriers include any

35   carrier that does not itself induce the production of

antibodies harmful to the individual receiving the composition. Suitable carriers are typically large, slowly metabolized macromolecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids,

5 polymeric amino acids, amino acid copolymers; and inactive virus particles. Such carriers are well known to those of ordinary skill in the art.

Preferred adjuvants to enhance effectiveness of the composition include adjuvants listed below with

10 formulation for one human vaccine dose of 0.5 ml. the dosages of components described below are preferred dosages; variations in dosages that do not affect the function of the adjuvant are also encompassed by the present invention as can be readily determined by the

15 person skilled in the art.

Adjuvant A, described in WO 96/33739, with the formulation 0.25 mg cholesterol, 1 mg dioleoyl phosphotidylcholine, 50 ug 3D-MPL, and 50 ug QS21 and consisting of small liposomes wherein the QS21 and the

20 3D-MPL are in the membranes of the liposomes;

Adjuvant B, described in U.S. Patent no. 6,146,632, with the formulation 10.68 mg squalene, 11.86 mg tocopherol, 4.85 mg Tween 80, 50 ug 3D-MPL, and 50 ug QS21 and consisting of an oil-in water

25 emulsion comprising the squalene and alpha-tocopherol, the emulsion being in admixture with the QS21 and 3-DPML;

Adjuvant C, described in WO 96/33739, with the formulation 0.25 mg cholesterol, 1 mg dioleoyl

30 phosphotidylcholine, 50 ug 3D-MPL, 50 ug QS21 and 0.5 mg AlOH$_3$ and consisting of small liposomes wherein the saponin (QS21) and the LPS-derivative (3D-MPL) are in the membranes of the liposomes and wherein the liposomes and the antigen are absorbed onto a metallic

35 salt particle carrier (AlOH$_3$);

Adjuvant D, with the formulation 0.5 mg $AlOH_3$, 500 ug of unmethylated immunostimulatory oligonucleotide CpG described in WO 96/02555 (CpG = 5'-tcg tcg ttt tgt cgt ttt gtc gtt) (SEQ ID NO:8)

5   where antigen and immunostimulant (CpG) are absorbed onto a metallic salt particle carrier ($AlOH_3$);

Adjuvant E, described in WO 96/33739, with the formulation 0.25 mg cholesterol, 1 mg dioleoyl phosphotidylcholine, 50 ug QS21, and 0.5 mg $AlOH_3$,

10   consisting of small unilamellar vesicles wherein the saponin (QS21) is in the membranes of the vesicles and wherein the vesicles and the antigen are absorbed onto a metallic salt particle $AlOH_3$.

All documents cited herein are hereby incoporated

15   by reference thereto.

The immunogenic compositions typically will contain pharmaceutically acceptable vehicles, such as water, saline, glycerol, ethanol, etc. Additionally, auxiliary substances, such as wetting or emulsifying

20   agents, pH buffering substances, preservatives, and the like, may be included in such vehicles.

Typically, the immunogenic compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or

25   suspension in, liquid vehicles prior to injection may also be prepared. The preparation also may be emulsified or encapsulated in liposomes for enhanced adjuvant effect. The $MSP-1_{42}$ proteins of the invention may also be incorporated into Immune Stimulating

30   Complexes together with saponins, for example QuilA (ISCOMS).

Immunogenic compositions used as vaccines comprise a `sufficient amount` or `an immunologically effective amount` of the proteins of the present

35   invention, as well as any other of the above mentioned

components, as needed. `Immunologically effective
amount`, means that the administration of that amount
to an individual, either in a single dose or as part
of a series, is effective for treatment, as defined

5   above. This amount varies depending upon the health
and physical condition of the individual to be
treated, the taxonomic group of individual to be
treated (e.g. nonhuman primate, primate, etc.), the
capacity of the individual's immune system to

10   synthesize antibodies, the degree of protection
desired, the formulation of the vaccine, the treating
doctor's assessment of the medical situation, the
strain of malaria infection, and other relevant
factors. It is expected that the amount will fall in a

15   relatively broad range that can be determined through
routine trials. Usually, the amount will vary from
0.01 to 1000 ug/dose, more particularly from about 1.0
to 100 ug/dose most preferably from about 10 to 50
ug/dose.

20   The proteins may also serve as vaccine carriers
to present homologous (e.g. other malaria antigens,
such as EBA-175 or AMA-1) or heterologous (non-
malaria) antigens. In this use, the proteins of the
invention provide an immunogenic carrier capable of

25   stimulating an immune response to other antigens. The
antigen may be conjugated either by conventional
chemical methods, or may be cloned into the gene
encoding MSP-1$_{42}$ fused to the 5'end or the 3' end of
the MSP-1$_{42}$ gene. The vaccine may be administered in

30   conjunction with other immunoregulatory agents.
     The compounds of the present invention can be
formulated according to known methods to prepare
pharmaceutically useful compositions, whereby these
materials, or their functional derivatives, are

35   combined in admixture with a phamaceutically

acceptable carrier vehicle. Suitable vehicles and
their formulation, inclusive of other human proteins,
e.g., human serum albumin, are described, for example,
in *Remington's Pharmaceutical Sciences* (16th ed.,
5   Osol, A. ed., Mack Easton Pa. (1980)). In order to
form a pharmaceutically acceptable composition
suitable for effective administration, such
compositions will contain an effective amount of the
above-described compounds together with a suitable
10   amount of carrier vehicle.

Additional pharmaceutical methods may be employed
to control the duration of action. Control release
preparations may be achieved through the use of
polymers to complex or absorb the compounds. The
15   controlled delivery may be exercised by selecting
appropriate macromolecules (for example polyesters,
polyamino acids, polyvinyl, pyrrolidone,
ethylenevinylacetate, methylcellulose,
carboxymethylcellulose, or protamine sulfate) and the
20   concentration of macromolecules as well as the method
of incorporation in order to control release. Another
possible method to control the duration of action by
controlled release preparations is to incorporate the
compounds of the present invention into particles of a
25   polymeric material such as polyesters, polyamino
acids, hydrogels, poly(lactic acid) or ethylene
vinylacetate copolymers. Alternatively, instead of
incorporating these agents into polymeric particles,
it is possible to entrap these materials in
30   microcapsules prepared, for example, interfacial
polymerization, for example, hydroxymethylcellulose or
gelatin-microcapsules and poly(methylmethacylate)-
microcapsules, respectively, or in colloidal drug
delivery systems, for example, liposomes, albumin
35   microspheres, microemulsions, nanoparticles, and

nanocapsules or in macroemulsions. Such techniques are disclosed in *Remington's Pharmaceutical Sciences* (1980).

5   Administration of the compounds, whether antibodies or vaccines, disclosed herein may be carried out by any suitable means, including parenteral injection (such as intraperitoneal, subcutaneous, or intramuscular injection), *in ovo* injection of birds, orally, or by topical

10   application of the antibodies (typically carried in a pharmaceutical formulation) to an airway surface. Topical application of the antibodies to an airway surface can be carried out by intranasal administration (e.g., by use of dropper, swab, or

15   inhaler which deposits a pharmaceutical formulation intranasally). Topical application of the antibodies to an airway surface can also be carried out by inhalation administration, such as by creating respirable particles of a pharmaceutical

20   formulation (including both solid particles and liquid particles) containing the antibodies as an aerosol suspension, and then causing the subject to inhale the respirable particles. Methods and apparatus for administering respirable particles of

25   pharmaceutical formulations are well known, and any conventional technique can be employed. Oral administration may be in the form of an ingestable liquid or solid formulation.

    The treatment may be given in a single dose

30   schedule, or preferably a multiple dose schedule in which a primary course of treatment may be with 1-10 separate doses, followed by other doses given at subsequent time intervals required to maintain and or reinforce the response, for example, at 1-4

35   months for a second dose, and if needed, a

subsequent dose(s) after several months. Examples
of suitable treatment schedules include: (i) 0, 1
month and 6 months, (ii) 0, 7 days and 1 month,
(iii) 0 and 1 month, (iv) 0 and 6 months, or other
schedules sufficient to elicit the desired
responses expected to reduce disease symptoms, or
reduce severity of disease.

The present invention also provides kits which
are useful for carrying out the present invention.
The present kits comprise a first container means
containing the above-described antibodies. The kit
also comprises other container means containing
solutions necessary or convenient for carrying out the
invention. The container means can be made of glass,
plastic or foil and can be a vial, bottle, pouch,
tube, bag, etc. The kit may also contain written
information, such as procedures for carrying out the
present invention or analytical information, such as
the amount of reagent contained in the first container
means. The container means may be in another
container means, e.g. a box or a bag, along with the
written information.

The present invention also relates to a method
for *in vitro* diagnosis of malaria antibodies present
in a biological sample, comprising at least the
following steps

(i) contacting said biological sample with a
composition comprising any of the MSP-1_{42} peptides as
defined above, preferably in an immobilized form under
appropriate conditions which allow the formation of an
immune complex, wherein said peptide or protein can be
a biotinylated peptide or protein which is covalently
bound to a solid substrate by means of streptavidin or
avidin complexes,

(ii) removing unbound components,

(iii) incubating the immune complexes formed with heterologous antibodies, with said heterologous antibodies having conjugated to a detectable label

5   under appropriate conditions,

(iv) detecting the presence of said immune complexes visually or mechanically (e.g. by means of densitometry, fluorimetry, colorimetry).

The present invention also relates to a kit for

10   determining the presence of malaria antibodies, in a biological sample, comprising:

at least one peptide or protein composition as defined above, possibly in combination with other polypeptides or peptides from *Plasmodium* or other

15   types of malaria parasite, with said peptides or proteins being preferentially immobilized on a solid support, more preferably on different microwells of the same ELISA plate, and even more preferentially on one and the same membrane strip,

20   a buffer or components necessary for producing the buffer enabling binding reaction between these polypeptides or peptides and the antibodies against malaria present in the biological sample,

means for detecting the immune complexes formed

25   in the preceding binding reaction,

possibly also including an automated scanning and interpretation device for inferring the malaria parasite present in the sample from the observed binding pattern.

30   The immunoassay methods according to the present invention utilize MSP-1_{42} domains that maintain linear (in case of peptides) and conformational epitopes (proteins) recognized by antibodies in the sera from individuals infected with a malaria parasite. The MSP-

35   1_{42} antigens of the present invention may be employed

in virtually any assay format that employs a known
antigen to detect antibodies. A common feature of all
of these assays is that the antigen is contacted with
the body component suspected of containing malaria
5   antibodies under conditions that permit the antigen to
bind to any such antibody present in the component.
Such conditions will typically be physiologic
temperature, pH and ionic strenght using an excess of
antigen. The incubation of the antigen with the
10   specimen is followed by detection of immune complexes
comprised of the antigen.

Design of the immunoassays is subject to a great
deal of variation, and many formats are known in the
art. Protocols may, for example, use solid supports,
15   or immunoprecipitation. Most assays involve the use
of labeled antibody or polypeptide; the labels may be,
for example, enzymatic, fluorescent, chemiluminescent,
radioactive, or dye molecules. Assays which amplify
the signals from the immune complex are also known;
20   examples of which are assays which utilize biotin and
avidin or streptavidin, and enzyme-labeled and
mediated immunoassays, such as ELISA assays.

The immunoassay may be, without limitation, in a
heterogeneous or in a homogeneous format, and of a
25   standard or competitive type. In a heterogeneous
format, the polypeptide is typically bound to a solid
matrix or support to facilitate separation of the
sample from the polypeptide after incubation. Examples
of solid supports that can be used are nitrocellulose
30   (e.g., in membrane or microtiter well form), polyvinyl
chloride (e.g., in sheets or microtiter wells),
polystyrene latex (e.g., in beads or microtiter
plates, polyvinylidine fluoride (known as
Immunolon.TM.), diazotized paper, nylon membranes,
35   activated beads, and Protein A beads. For example,

Dynatech Immunolon.TM.1 or Immunlon.TM. 2 microtiter plates or 0.25 inch polystyrene beads (Precision Plastic Ball) can be used in the heterogeneous format. The solid support containing the antigenic

5 polypeptides is typically washed after separating it from the test sample, and prior to detection of bound antibodies. Both standard and competitive formats are know in the art.

In a homogeneous format, the test sample is

10 incubated with the combination of antigens in solution. For example, it may be under conditions that will precipitate any antigen-antibody complexes which are formed. Both standard and competitive formats for these assays are known in the art.

15 In a standard format, the amount of malaria antibodies in the antibody-antigen complexes is directly monitored. This may be accomplished by determining whether labeled anti-xenogeneic (e.g. anti-human) antibodies which recognize an epitope on

20 anti-malaria antibodies will bind due to complex formation. In a competitive format, the amount of malaria antibodies in the sample is deduced by monitoring the competitive effect on the binding of a known amount of labeled antibody (or other competing

25 ligand) in the complex.

Complexes formed comprising anti-malaria antibody (or in the case of competitive assays, the amount of competing antibody) are detected by any of a number of known techniques, depending on the format. For

30 example, unlabeled malaria antibodies in the complex may be detected using a conjugate of anti-xenogeneic Ig complexed with a label (e.g. an enzyme label).

In an immunoprecipitation or agglutination assay

35 format the reaction between the malaria antigens and

the antibody forms a network that precipitates from the solution or suspension and forms a visible layer or film of precipitate. If no anti-malaria antibody is present in the test specimen, no visible precipitate

5   is formed.

There currently exist three specific types of particle agglutination (PA) assays. These assays are used for the detection of antibodies to various antigens when coated to a support. One type of this

10   assay is the hemagglutination assay using red blood cells (RBCs) that are sensitized by passively adsorbing antigen (or antibody) to the RBC. The addition of specific antigen antibodies present in the body component, if any, causes the RBCs coated with

15   the purified antigen to agglutinate.

To eliminate potential non-specific reactions in the hemagglutination assay, two artificial carriers may be used instead of RBC in the PA. The most common of these are latex particles. However, gelatin

20   particles may also be used. The assays utilizing either of these carriers are based on passive agglutination of the particles coated with purified antigens.

The MSP-1_{42} proteins, peptides, or antigens of the

25   present invention will typically be packaged in the form of a kit for use in these immunoassays. The kit will normally contain in separate containers the MSP-1_{42} antigen, control antibody formulations (positive and/or negative), labeled antibody when the assay

30   format requires the same and signal generating reagents (e.g. enzyme substrate) if the label does not generate a signal directly. The MSP-1_{42} antigen may be already bound to a solid matrix or separate with reagents for binding it to the matrix. Instructions

(e.g. written, tape, CD-ROM, etc.) for carrying out the assay usually will be included in the kit.

Immunoassays that utilize the MSP-1$_{42}$ antigen are useful in screening blood for the preparation of a supply from which potentially infective malaria parasite is lacking. The method for the preparation of the blood supply comprises the following steps. Reacting a body component, preferably blood or a blood component, from the individual donating blood with MSP-1$_{42}$ proteins of the present invention to allow an immunological reaction between malaria antibodies, if any, and the MSP-1$_{42}$ antigen. Detecting whether anti-malaria antibody--MSP-1$_{42}$ antigen complexes are formed as a result of the reacting. Blood contributed to the blood supply is from donors that do not exhibit antibodies to the native MSP-1 antigens.

The present invention further contemplates the use of MSP-1$_{42}$ proteins, or parts thereof as defined above, for *in vitro* monitoring malaria infection or prognosing the response to treatment (for instance with chloroquine, mefloquine, Malarome) of patients suffering from malaria infection comprising:

incubating a biological sample from a patient with malaria infection with an MSP-1$_{42}$ protein or a suitable part thereof under conditions allowing the formation of an immunological complex,

removing unbound components,

calculating the anti-MSP-1$_{42}$ titers present in said sample (for example at the start of and/or during the course of therapy),

monitoring the natural course of malaria infection, or prognosing the response to treatment of said patient on the basis of the amount anti-MSP-1$_{42}$ titers found in said sample at the start of treatment and/or during the course of treatment.

Patients who show a decrease of 2, 3, 4, 5, 7, 10, 15, or preferably more than 20 times of the initial anti-MSP-1$_{42}$ titers could be concluded to be long-term, sustained responders to malaria therapy.

5   It is to be understood that smaller fragments of the above-mentioned peptides also fall within the scope of the present invention. Said smaller fragments can be easily prepared by chemical synthesis and can be tested for their ability to be used in an assay as

10   detailed above.

The present invention also relates to a kit for monitoring malaria infection or prognosing the response to treatment (for instance to medication) of patients suffering from malaria infection

15   comprising:

at least one MSP-1$_{42}$ peptide as defined above,

a buffer or components necessary for producing the buffer enabling the binding reaction between these proteins or peptides and the anti-MSP-1$_{42}$

20   antibodies present in a biological sample,

means for detecting the immune complexes formed in the preceding binding reaction,

possibly also an automated scanning and interpretation device for inferring a decrease of

25   anti-MSP-1$_{42}$ titers during the progression of treatment.

The present invention also relates to a serotyping assay for detecting one or more serological types or alleles of malaria parasite present in a

30   biological sample, more particularly for detecting antibodies of the different types or alleles of malaria parasites to be detected combined in one assay format, comprising at least the following steps:

(i) contacting the biological sample to be

35   analyzed for the presence of malaria antibodies of one

or more serological types, with at least one of the MSP-1$_{42}$ compositions as defined above, preferentially in an immobilized form under appropriate conditions which allow the formation of an immune complex,

5   (ii) removing unbound components,

        (iii) incubating the immune complexes formed with heterolagous antibodies, with said heterologous antibodies being conjugated to a detectable label under appropriate conditions,

10   (iv) detecting the presence of said immune complexes visually or mechanically (e.g. by means of densitometry, fluorimetry, calorimetry) and inferring the presence of one or more malaria serological types present from the observed binding pattern.

15   It is to be understood that the compositions of proteins or peptides used in this method are recombinantly expressed type-specific or allele-specific proteins or type-specific peptides.

        The present invention further relates to a kit

20 for serotyping one or more serological types or alleles of malaria parasite present in a biological sample, more particularly for detecting the antibodies to these serological types of malaria parasites comprising:

25   at least one MSP-1$_{42}$ protein or MSP-1$_{42}$ peptide, as defined above,

        a buffer or components necessary for producing the buffer enabling the binding reaction between these proteins or peptides and the anti-MSP-1 antibodies

30 present in a biological sample,

        means for detecting the immune complexes formed in the preceding binding reaction,

        possibly also an automated scanning and interpretation device for detecting the presence of

one or more serological types present from the
observed binding pattern.

The present invention also relates to the use of
a peptide or protein composition as defined above, for
5   immobilization on a solid support and incorporation
into a reversed phase hybridization assay, preferably
for immobilization as parallel lines onto a solid
support such as a membrane strip, for determining the
presence or the genotype of malaria parasite according
10   to a method as defined above. Combination with other
type-specific or allele-specific antigens from other
malaria parasites also lies within the scope of the
present invention.

The contents of all cited references (including
15   literature references, issued patents, published
patent applications, and co-pending patent
applications) cited throughout this application are
hereby expressly incorporated by reference.

Other features of the invention will become
20   apparent in the course of the following descriptions
of exemplary embodiments which are given for
illustration of the invention and are not intended to
be limiting thereof.

The following MATERIALS AND METHODS were used in
25   the examples that follow.

Construction of expression cassette pET(AT)PfMSP-
1$_{42}$ (3D7)

Molecular cloning and bacterial transformations
were performed as described (Sambrook et al.,
30   Molecular cloning: A Laboratory Manual, Cold Spring
Harbor Laboratory Press, 1989). The final expression
construct, pETATPfMSP-142 (3D7) Tolle et al., 1995,
Exp. Parasitol. 81, 47-54, was the product of a series
of subclonings, each successive construction reducing

the amount of expressed non-MSP-1 sequence. The MSP-1_{42} fragment was prepared by PCR of genomic DNA using the forward primer GGGGATCCATTGAGGGTCGTGGTACCATGGC AATATCTGTCACAATGG (SEQ ID NO:9) and the reverse primer

5 GTCGACTTAGGAACTGCAGAAAATACCGG (SEQ ID NO:10). The product was cloned into the expression vector pMAL-p (New England Biolabs) via the 5' *BamHI* and the 3' *SalI* site, sequenced (SEQ ID NO:4), and then subcloned into pET32a (Novagen, Madison, Wisconsin), creating pET-

10 Trx42, which contained the MSP-1_{42} gene fragment fused in-frame to the 3' end of the *E. coli* thioredoxin gene, trx. pET-Trx42 was digested with NdeI and religated to remove trx, creating pET(50)MSP-1_{42}. pET42A was created by digesting both the pET(50)MSP-

15 1_{42} vector and the DNA fragment GGGCATATGGCACACCATCATCATCATCATCCCGGGGGATCCGAC (SEQ ID NO: 11) with *NdeI* and *BamHI* and then ligating the two. The DNA fragment encoded six consecutive histidine residues and a short flexible linker sequence. To

20 avoid using ampicillin selection, tet was subcloned from pBR322 by digesting with *EcoRI* and *PflMI*, then blunt-ending and ligating it into the Bst1107 I site in pET42A creating pET-IEGR-MSP-1_{42}(AT). The final plasmid pET(AT)PfMSP-1_{42}(3D7) was created by removing

25 the residual Factor Xa cleavage site. This was accomplished by digesting pET-IEGR-MSP-1_{42}(AT) and the DNA fragment GGGCATATGGCACACCATCATCATCATCATCCCGGGGGAT CCGGTTCTGGTACCGAC (SEQ ID NO:12), with NdeI and KpnI and then ligating. 3D7 MSP-1_{42} was expressed from this

30 final construct with 17 non-MSP-1_{42} amino acids fused to the N-terminus.

Expression of MSP-1_{42}.

The expression host, BL21 DE3 (F- ompT hsdSB(rB-mB-) gal dcm (DE3)) was transformed with pETATPfMSP-

35 1_{42}(3D7). Fresh stationary phase cultures of

transformed bacteria were used to inoculate 1L shake flasks of Super Broth containing 15 ug/ml tetracycline, which were grown to 0.4 OD 600 at 37°C , cooled to temperatures ranging from 25-35°C and

5   induced with 0.1 mM IPTG. Cell pastes were collected in lysis buffer (10 mM $NaPO_4$, pH 6.2, 50 mM NaCl, 10 mM imidazole, 2 mM $MgCl_2$, 50 U/ml benzonase) at a paste to buffer ratio of 1:3 w/v. Cells were lysed by microfluidization (Microfluidics) in one pass, NaCl

10 was added to a final concentration of 500 mM. The temperature of the sample was maintained below 10°C at all times. Tween-80, 1.0% w/v (final concentration), was added and the lysate was centrifuged at 27,666 x g for 1 hr at 4°C. Pellets and supernates were

15 evaluated by immunoblotting.

Bulk Fermentation and expression of MSP-1$_{42}$ (3D7).

A 300 L fermentor of Super Broth supplemented with 15 ug/ml tetracycline was inoculated with three L of fresh stationary phase culture in accordance with

20 Batch Production Record (BPR)-305-00. Fermentation continued until an OD 600 = 4.0 - 6.0 was reached. The fermentor was cooled to 25°C prior to induction with 0.1 mM IPTG. Three hours following induction, cells were harvested by centrifugation at 15,000 rpm at 3

25 L/min. The cell paste was stored at -80°C in the WRAIR Department of Biologics Research, Pilot Bioproduction Facility.

GMP Purification of *E. coli* expressed MSP-1$_{42}$ (3D7), BPR-335-02. Cell paste was lysed as described

30 above. Tween-80 was added to 1% w/v (final), and the lysate was centrifuged at 27,666 x g for 1 hr at 4°C. The clarified lysate was collected and placed on ice. All further steps were carried out at 4°C.

Ni^{+2} NTA Superflow (Qiagen, Germany): A column
with a 6:1 w/v cell paste to resin ratio, was
equilibrated with 10 mM NaPO$_4$, pH 6.2, 500 mM NaCl, 10
mM imidazole (Ni-buffer) supplemented with 0.5% Tween

5   80 (w/v). The clarified lysate was applied at a flow
rate of 30 ml/min and the column washed with 1.1
volumes of Ni-buffer containing 0.5% Tween-80. The
column was then washed with 30 volumes of Ni-buffer
containing 0.5% Tween 80 (w/v); 20 volumes of 10 mM

10   NaPO$_4$, pH 6.2, 75 mM NaCl, 10 mM imidazole; and 15
volumes of 10 mM NaPO$_4$, pH 8.0, 75 mM NaCl, 20 mM
imidazole. MSP-1$_{42}$ was eluted with 10 volumes 10 mM
NaPO$_4$, pH 8.0, 75 mM NaCl, 160 mM imidazole, and was
diluted with an equal volume of 10 mM NaPO$_4$, 75 mM

15   NaCl, pH 8.0, and 0.4% Tween 80.

Q ion exchange chromatography: A Toyopearl
SuperQ 650 M (TosoHaas) column (cell paste: resin
ratio = 3:1 w/v), was equilibrated with 10 mM NaPO$_4$,
pH 8.0, 75 mM NaCl, 80 mM imidazole, 0.2% Tween 80 (Q-

20   buffer). The diluted sample was applied at a flow
rate of 30 ml/min and washed with one volume of Q-
buffer, which was combined with the flow through to
pool the MSP-1$_{42}$, giving a final volume of this pool
equal to 50 Q column volumes. This sample was diluted

25   with an equal volume of 10 mM NaPO$_4$, pH 6.0, 0.4%
Tween 80 (v/v), and the pH of the sample was adjusted
to pH 6.0 with 6N HCl.

CM ion exchange chromatography: A CM 650 M
(TosoHaas) column (cell paste:resin ratio = 2:1) was

30   equilibrated with 10 mM NaPO$_4$, pH 6.0, 35 mM NaCl,
0.2% Tween 80 (CM-equilibration buffer) and the sample
was applied at a flow rate of 30 ml/min. The column
was washed with 6 volumes of CM-equilibration buffer,
followed by 10 volumes of 10mM NaPO$_4$, pH 7.0, 100 mM

35   NaCl, 0.02% Tween 80 (v/v). The MSP-1$_{42}$ was eluted

with 10 mM $NaPO_4$, pH 7.2, 250 mM NaCl in three column volumes.

SDS-PAGE and Immunoblotting. Protein samples were separated under reducing (10% 2-mercaptoethanol) or nonreducing conditions by SDS-PAGE with Tris-Glycine buffering (Invitrogen). Protein was detected by Coomassie Blue R250 staining. Immunoblotting was perfomed with nitrocellulose membranes (Invitrogen) blocked using 5% nonfat dry milk and 0.1% Tween 20 in PBS, pH 7.4. Blots were probed with polyclonal rabbit anti-MSP-1$_{42}$ antibodies or mAbs diluted into phosphate buffered saline, pH 7.4 containing 0.1% Tween 20. This buffer was also used for washing. The second antibodies were alkaline phosphatase-conjugated anti-rabbit IgG or anti-mouse IgG (H+L) (Promega, Madison, Wisconsin) and reactions were detected with nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Sigma Chemicals, St. Louis, Missouri) in 100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris-HCl, pH 9.5. mAbs used for evaluation of structure included 2.2 (Mackay et al., 1985, Embo J. 4, 3823-3829), 12.8 (Conway et al, 1991, Parasitology 103, 1-6), 7.5 (McBride et al., 1982, Science 217, 254-257), 12.10 (Blackman et al., 1990, supra), 5.2 (Chang et al., 1988, Exp. Parasitol. 67, 1-11), and 7F1 (Lyon et al., 1987, J. Immunol. 138, 895-901).

Antigen Stability Studies

Stability studies were conducted for 18 months on MSP-1$_{42}$ stored at 4°C, -20°C, and -80°C and for lyophilized product stored at -20°C. Stability was evaluated by Commassie Blue staining and immunoblotting of SDS PAGE gels run under non-reduced and reduced conditions.

Vaccine Preparation

The CM eluate was concentrated two-fold to 0.5 mg/ml, the buffer was exchanged with 10 volumes of phosphate buffered saline by diafiltration and the protein was sterilized by filtration through a

5   Millipak 60 0.22-um filtration unit. Final bulk antigen (FMP1) was stored at -80°C. To prepare the antigen for use with adjuvant ADJUVANT B (GlaxoSmithKline Biologicals, Rixensart, Belgium), 118.5 ml of sterile purified FMP1 was mixed with 236

10 ml of 50 mM Na Phosphate, 101 ml of 15.75% lactose, and 0.5 ml Tween-80, producing formulated antigen at a concentration of 118.5 mg/ml. Formulated FMP1 was sterile filtered with Millipak 40 0.22-um filtration unit and added to 3 ml vials for lyophilization. The

15 vials were sealed with Lyo stoppers and metal crimps (BPR-334-01, Lot 0678)

Vaccine Potency

Female Balb/C mice were immunized subcutaneously with 100 ul of vaccine. Potency studies were

20 performed with 1.0, 0.3 and 0.1 ug of MSP-1$_{42}$ in Adjuvant B (GlaxoSmithKline Biologicals, Rixensart, Belgium). The 10 ug dose was prepared with 10 ug of antigen and 100 ul of Adjuvant B and was used to make the 1.0 and 0.1 ug doses by diluting into 0.9% saline.

25 Mice were primed and then bled and boosted four- weeks later, and bled again 2 weeks following boosting. Sera were analyzed by ELISA and results are reported in ELISA units, or the serum dilution that gives an absorbance of 1 OD 405 (Stoute et al., 1997, N. Engl.

30 J. Med. 336, 86-91).

Seroconversion occurred if the following condition was met:(ELISA Units-3SD)post-vaccination - (ELISA Units+3SD)pre-vaccination > 0

Safety and Immunogenicity

Rhesus monkeys were vaccinated intramuscularly with 50 ug of antigen formulated with ADJUVANT B or alum. Monkeys were boosted one, three, five, and seven months after priming and sera were collected

5   prior to and two weeks following each immunization.

Serology

Sera were analyzed by IFA against methanol fixed 3D7 strain *P. falciparum* schizont-infected erythrocytes (Lyon et al., 1987, supra) and by kinetic

10   ELISA. For this, the MSP-1$_{42}$ capture antigen was diluted in PBS at pH 7.4 and coated at 0.4 pmoles /well overnight at 4°C, and wells were blocked with CaseinBlock (Pierce). Sera were diluted 1:50,000 (ADJUVANT B) or 1:8,000 (Alum) in CaseinBlock and

15   reacted for 1 h at room temperature, followed by reaction with alkaline phosphatase-conjugated rabbit anti-human IgG (H&L) (Promega) diluted 1:250 in CaseinBlock also for 1 hr at room temperature. Detection of p-nitrophenyl phosphate substrate

20   conversion to product was measured at 5 min intervals for 30 min. The slope of the line was calculated by linear regression and R2 was at least 0.99 for each analysis.

25   Inhibition of Parasite Invasion

Rabbits were vaccinated four times subcutaneously with FMP1 in Freund's adjuvants and sera were collected three weeks after the final immunization. The IgG fractions of pre-immune and post-immune sera

30   were prepared with Protein-G chromatography and quantified by using the Bradford Protein Determination assay (Pierce). For the invasion inhibition assay (Chulay et al., 1981, Am. J. Trop. Med. Hyg. 30, 12-19), the IgG fractions were dialyzed against RPMI 1640

adjusted to pH 7.4 with NaOH and added to settled 100
ul cultures of synchronous *P. falciparum* (3D7 strain)
schizont-infected erythrocytes (3-5 nuclei) at 2%
hematocrit and 0.25% parasitemia. In some

5   experiments, native FMP1 or reduced and alkykated
FMP1, dialyzed as above, were added as specificity
controls to reverse the activity of inhibitory
antibodies.

Example 1

10   The gene fragment containing *P. falciparum* 3D7
strain MSP-1$_{42}$ was cloned into pET32a (Figure 1A) with
several modifications. These included adding the gene
for tetracycline, removing the trx gene and other
elements, and adding a hexa-histidine affinity tag,

15   which contained 17 non-MSP-1$_{42}$ amino acids fused to the
N-terminus of MSP-1$_{42}$ (Figure 1B). Our primary
objective of expressing MSP-1$_{42}$ as a soluble protein in
E. coli cytoplasm was achieved by systematically
varying IPTG concentration and induction temperature.

20   IPTG at 0.1 mM induced maximal protein synthesis and
induction at 25°C was required to express soluble
protein (data not shown). The optimal biomass density
for induction in 10L fermentors was shown to be 4-6
OD600. These conditions were used to prepare GMP cell

25   paste in a 300 L fermentor, and this paste was used to
develop the purification process. The MSP-1$_{42}$ was
purified by three chromatographic steps. After
centrifugation, cleared lysates were applied to a Ni^{+2}-
NTA Superflow resin for affinity purification (Figure

30   2A, lane 1). This step removed most of the endotoxin,
which depended on extensive washing of bound protein
at low pH (pH 6.2) and high sodium chloride (500 mM).
After this step, the protein was greater than 50% pure
by densitometry (arrows show MSP-1$_{42}$ related bands).

35   MSP-1$_{42}$ was purified to greater than 95% purity by

chromatography on a Q-anion exchanger followed by a
CM-cation exchanger (Figure 2A, lanes 2 and 3
respectively). The Final Bulk Antigen (FMP1) was
further characterized by SDS-PAGE under non-reducing
5   and reducing conditions (Figure 2B, lanes 1 and 2,
respectively) and by immunoblotting with rabbit anti-
E. coli antibody (Figure 2C, lane 3) and MSP-1
specific mAbs. The MSP-1$_{33}$ specific mAb 7F1 reacted
with two proteins migrating at 36kD and 38kD (Figure
10 2C, lane 1), but the MSP-1$_{19}$ specific mAb 12.10 (Figure
2C, lane 2) did not, nor did any of the other MSP-1$_{19}$
specific mAbs. Full-length MSP-1$_{42}$ (see Figure 2) and
all of the higher molecular weight aggregates were
reactive against all the MSP-1 specific mAbs used.
15 Long term stability studies showed that the FMP1 was
stable when stored for 18 months at -80°C but not when
stored at 4°C (degradation) or -20°C (aggregation)
(Figure 3, lanes 1-3, respectively), compare with
Figure 2B (lane 1).
20   **Example 2**
Vaccine potency studies were conducted in Balb/C
mice immunized with FMP1 formulated for use with
adjuvant ADJUVANT B. At the 0.3 ug and 1.0 ug doses,
respectively, all mice seroconverted following one
25 immunization. At the 0.1 ug dose 50% of the mice
seroconverted following the first immunization; all
seroconverted after the second (data not shown).
Safety and immunogenicity of the product was assessed
in Rhesus monkeys immunized up to five times with 50
30 ug doses of FMP1/ADJUVANT B (n=8) or FMP1/alum (n=6).
No adverse local responses were observed and all
biochemical and hematological laboratory tests were
normal for both groups (not shown). FMP1/ADJUVANT B
induced malaria parasite reactive IFA titers that
35 increased to 1:28,000 by two weeks following the

second immunization and maintained this level through
the third immunization (Figure 4, right ordinate).
The fourth immunization induced a brief increase in
titer, which returned to the 1:28,000 base within six

5 weeks. The fifth immunization induced a response that
was similar to the fourth. FMP1/alum induced IFA titer
that paralleled those induced by FMP1/ADJUVANT B but
were six-fold lower. FMP1/ADJUVANT B induced $MSP-1_{42}$-
specific ELISA-reactive antibodies that increased to

10 2100 OD/min by two weeks following the second
immunization and gradually increase to a maximum of
2800 OD/min after the fourth immunization (Figure 4,
left ordinate). The geometric mean antibody level
after five immunizations was lower than after four,

15 but this difference was not significant. By four
weeks after each immunization antibody levels fell
about 30% but stabilized after the third immunization.
FMP1/alum induced ELISA-reactive antibodies levels
roughly paralleled those induced by FMP1/ADJUVANT B

20 but were about ten-fold lower. FMP1 in Freund's
adjuvants induced invasion inhibitory IgG antibodies.
The inhibition was titratible (Figure 5) and was
completely reversed by adding competing soluble FMP1
at a final concentration of 17 ug/ml and partially

25 reversed by adding reduced and alkylated
FMP1(approximately 50% reversal) at the same
concentration (data not shown).

Example 3

FMP1 combined with the adjuvants ADJUVANT A,

30 ADJUVANT B ADJUVANT C, ADJUVANT E, Adjuvant D, and
Alum were assessed for safety and immunogenicity in
Rhesus monkeys at the human dose of 50 ug/injection.
Studies were conducted in adult *Macaca mulatta*
housed at the Armed Forces Research Institute of

35 Medical Sciences (AFRIMS), Bangkok, Thailand. Monkeys

were screened to exclude animals in poor health or with previous exposure to malaria, and were monitored for at least 6 weeks prior to the start of the study. Animals were randomly assigned to treatment groups,

5   and vaccines were administered in a blinded standardized fashion while animals were under ketamine anesthesia.

Animals received intramuscular injections of FMP1 (50 ug) combined with adjuvant at 0, 4, 12 weeks.

10   The attending veterinarian who conducted clinical and laboratory evaluations assessed vaccine safety. Blood was obtained for CBC and serum chemistries prior to administration and at 24, 48, and 72 hours immediately after each immunization. Local reactogenicity was

15   monitored at the time of immunization, day 1, day 2, day 3, and day 14 post-immunization.

Safety

The following safety data apply to FMP1 combined with ADJUVANT B. The same data are available for the

20   other adjuvants as well.

Clinical evaluations before and after each immunization revealed minimal local and no systemic toxicities. Reactogenicity of FMP1 in ADJUVANT B was assessed in each Rhesus monkey using the following

25   criteria: 1) skin warmth or calor; 2) skin erythema or rubor; 3) skin swelling or edema; 4) muscle induration; 5) muscle necrosis. The intensity of the reactions were scored as follows: 0 = none; 1 = slight or mild reaction; 2 = moderate reaction; 3 = marked or

30   severe reaction. The mean prevalence (Table 2A) and mean intensity (Table 2B) of local reactogenicity is given in the tables below. Erythema, skin swelling, and muscle induration were limited to the site of inoculation and resolved in all monkeys by 14 days

35   post immunization. There were no cases of muscle

necrosis. The intensity of muscle induration
diminished daily from post immunization day 1 through
day 3, and multiple immunizations did not increase the
risk of adverse reactions. Behavior, activity, and
5   food consumption remained normal.

Table 2A

Mean Prevalence of Reactogenicity- mean % positive (n = 8 Rhesus)							
Immuni-zation Number		Post-immuni-zation day	Skin warmth/ calor	Skin erythema/ rubor	Skin swelling/ edema	Muscle induration	Muscle necrosis
1	10-Aug-99	-	0%	0%	0%	0%	0%
	11-Aug-99	1	50%	12.5%	37.5%	87.5%	0%
	12-Aug-99	2	0%	0%	12.5%	75%	0%
	13-Aug-99	3	0%	0%	0%	12.5%	0%
	24-Aug-99	14	0%	0%	0%	0%	0%
2	7-Sep-99	-	0%	0 of 8	0 of 8	0 of 8	0%
	8-Sep-99	1	12.5%	87.5%	100%	100%	0%
	9-Sep-99	2	0%	50%	100%	100%	0%
	10-Sep-99	3	0%	12.5%	87.5%	100%	0%
	21-Sep-99	14	0%	0%	0%	0%	0%
3	9-Nov-99	-	0 of 8	12.5%	2 of 8	0 of 8	0%
	10-Nov-99	1	37.5%	25%	75%	100%	0%
	11-Nov-99	2	12.5%	0%	37.5%	100%	0%
	12-Nov-99	3	0%	0%	0%	75%	0%
	23-Nov-99	14	0%	0%	0%	0%	0%

10

Table 2B

Immuni-zation Number		Post-immuni-zation day	Skin warmth/ calor	Skin erythema/ rubor	Skin swelling/ edema	Muscle induration	Muscle necrosis
1	10-Aug-99	-	0	0	0	0	0
	11-Aug-99	1	1	1	1.3	1.3	0
	12-Aug-99	2	0	0	1	1	0
	13-Aug-99	3	0	0	0	1	0
	24-Aug-99	14	0	0	0	0	0
2	7-Sep-99	-	0	0	0	0	0
	8-Sep-99	1	2	1.6	3	2.9	0

	9-Sep-99	2	0	1.8	3	2.4	0
	10-Sep-99	3	0	1	2.6	2.3	0
	21-Sep-99	14	0	0	0	0	0
3	9-Nov-99	-	0	0	0	0	0
	10-Nov-99	1	1	1.5	1.3	2.1	0
	11-Nov-99	2	1	0	1	1.6	0
	12-Nov-99	3	0	0	0	1.5	0
	23-Nov-99	14	0	0	0	0	0

Mean laboratory values for the entire group
(Tables 3 and data not shown) indicated no significant

5   abnormalities or trends in hematologic or biochemical
laboratory tests. Quantitative platelet counts varied
before and after immunization. This variation occurred
both within individual Rhesus monkeys and between
Rhesus. All blood prior to December 1999 was collected

10   in heparin anti-coagulant, and we suspected that the
platelet count variation was attributed to the use of
the heparin anticoagulant. All Rhesus in the study had
blood samples collected in mid December 1999 using
both heparin and EDTA anticoagulants and quantitative

15   platelet counts determined at 2 locations in Bangkok
Thailand. AFRIMS veterinary pathologists read all
blood smears from August, September, and November and
the platelet counts were read as adequate, increased,
or decreased. It was decided that all further

20   hematologic (CBC) laboratory assays would be collected
in EDTA. The January 2000 samples reflect this change
in blood collection procedure with normal platelet
counts observed in all Rhesus immunized with FMP1 and
ADJUVANT B days 1, 2, and 3 following a 4th

25   immunization. In addition, all blood smears at each
time point reviewed after immunizations 1, 2, and 3
had adequate platelet counts (data not shown).

Immunogenicity

Humoral Immunity: FMP1 combined with ADJUVANT A or ADJUVANT B induced similar levels of ELISA (Table 4A) and IFA (Table 4B) reactive antibody and were significantly more potent than the other adjuvant combinations. FMP1 combined with Alum was the least immunogenic by ELISA after two immunizations, but was not different from ADJUVANT C, ADJUVANT E and Adjuvant D by ELISA after the third immunization. FMP1 combined with Alum or with ADJUVANT C induced similar levels of IFA reactive antibody and induced significantly more IFA reactive antibody than FMP1 combined with ADJUVANT E or Adjuvant D.

Cellular Immunity: FMP1 combined with ADJUVANT A or ADJUVANT B induced different cytokine response profiles (Table 5). Both vaccines induced comparable levels of IFN-γ response after the 3rd dose, however, the IL-5 response was greatly suppressed in animals receiving FMP1 combined with ADJUVANT A. Duration of T cell response data indicate that IFN-γ response generated by FMP1 combined with both ADJUVANT A and ADJUVANT B persists at least 24 weeks after vaccination. The difference on Th1/Th2 polarization of FMP1 formulated with ADJUVANT B and ADJUVANT A was not anticipated.

Table 3: Mean Biochemical Laboratory Test Values

	Immunization #1 August				Immunization #2 September				Immunization #3 November			
	5	11	12	13	7	8	9	10	9	10	11	12
BUN (mg/ml)												
MEAN	19.7	17.3	18.1	19.0	20.0	16.9	20.6	17.9	17.9	18.5	16.3	16.4
s.d.	3.5	3.4	2.4	3.7	2.9	2.6	2.2	2.9	2.9	1.1	2.4	1.9
CREATININE (mg/ml)												
MEAN	0.76	0.81	0.80	0.81	0.80	0.83	0.75	0.81	0.88	0.90	0.83	0.86
s.d	0.07	0.04	0.11	0.06	0.09	0.12	0.08	0.11	0.18	0.09	0.10	0.12
AST (U/l)												
MEAN	23.9	80.8	67.9	61.9	27.4	47.0	50.1	47.6	27.8	68.8	57.3	46.0
s.d.	3.7	38.9	22.5	24.4	4.1	13.6	20.3	17.1	4.5	23.3	18.5	14.9
ALT (U/l)												
MEAN	28.0	50.9	50.1	56.3	32.8	37.3	39.5	43.9	38.4	41.5	47.0	51.5
s.d.	9.1	16.0	15.2	17.1	13.3	13.6	12.7	12.9	10.6	13.7	12.8	13.5
CPK (U/l)												
MEAN	301	1192	879	923	256	791	671	470	307	2443	915	520
s.d.	239	550	684	657	208	356	298	237	368	1039	721	221

Table 4A : Rhesus Humoral Responses to FMP1 formulated with various adjuvants

		Week 0*	Week 2	Midpoint ELISA Tier Week 4*	Week 6	Week 13*	Week 15
ADJUVANT A	GMT	13	1402	1824	52161	9291	74348
	95% CI	17	1413	1171	15783	4088	20937
ADJUVANT B	GMT	7	2439	3929	87096	25574	98458
	95% CI	2	657	2164	24748	6797	42826
ADJUVANT C	GMT	27	325	715	31654	2576	25120
	95% CI	101	597	714	15885	633	9183
ADJUVANT E	GMT	6	80	186	25360	2698	35945
	95% CI	3	155	234	9517	915	10550
ADJUVANT D	GMT	5	245	392	16899	1799	23647
	95% CI	24	315	259	16810	2113	12386
Alum	GMT	8	64	98	5501	751	10969
	95% CI	21	129	118	7168	865	11011

*Immunization time point

Table 4B

| | | Endpoint IFA Titer | | | | | |
		Week 0*	Week 2	Week 4*	Week 6	Week 13*	Week 15
ADJUVANT A	GMT	0	4000	1308	26251	11888	43069
	95% CI	0	2079	7492	5410	6372	11853
ADJUVANT B	GMT	0	3668	4362	26909	19027	26909
	95% CI	0	1283	1793	5132	6584	5132
ADJUVANT C	GMT	0	24	917	9514	2594	10375
	95% CI	488	257	849	6458	0	6187
ADJUVANT E	GMT	14	12	15	144	31	257
	95% CI	3	4	6	98	29	131
ADJUVANT D	GMT	50	54	74	675	169	844
	95% CI	13	19	21	319	66	238
Alum	GMT	0	316	794	2828	1414	4490
	95% CI	0	470	0	4158	839	3719

*Immunization time point

Table 5 : Polarization by ADJUVANT A of Immune

Rosponses to FMP1

Adjuvant	IFN-γ/IL-5
ADJUVANT A	4.2
ADJUVANT B	0.75
ADJUVANT C	2.3
ADJUVANT E	0.77
Adjuvant D	5.0
Alum	0.53

5 **Example 4**

A Phase I dose escalation clinical trial of the
recombinant Plasmodium falciparum malaria vaccine
candidate FMP1/ADJUVANT B was recently completed at
the Walter Reed Army Institute of Research (WRAIR) in
10 Silver Spring, MD in 15 adult human volunteers to
assess safety, reactogenicity, and immunogenicity.
This vaccine was created by researchers at the WRAIR
and manufactured at the Pilot Bioproduction Facility
at WRAIR. In the initial Phase I clinical trial
15 conducted during 4th quarter 2000 – 1st quarter 2001,
three groups of 5 volunteers were immunized with 1/5th
dose, 1/2 dose, or full dose of vaccine at 0, 1, and 3
months. Tables 1 and 2 below summarize the
demographics of the study population.
20

Safety and Adverse Events:

Local and general systemic adverse events were
assessed at 6 time points following each immunization,
and blood tests to evaluate hematologic, renal, and
25 hepatic abnormalities were performed before and after
each immunization. The vaccine proved safe in all 15
volunteers with NO serious adverse events or clinical
laboratory abnormalities noted. There were no drop-
outs from the vaccine trial. All adverse events were
30 graded according to severity. There were no Serious

Adverse Events (SAE's) requiring hospitalization nor
were there any grade 3 adverse events as defined
below. Grade "3" = Adverse experience which prevents
normal everyday activities and necessitates a

5 corrective therapy. The specific occurances by subject
and by dose of all adverse events are summarized in
tables 3-7 below. In summary, the most frequent
adverse events was minimal pain at the site of vaccine
injection which disappeared by 24 hours post-

10 inoculation.

Immunogenicity:

ELISA:

    The vaccine was extremely potent in inducing
high-titer antibody responses in all volunteers as

15 assessed by ELISA (enzyme-linked immunoabsorbent
assay). The table below summarizes the mean, standard
deviation, and geometric mean antibody titers for each
of the three vaccine groups.

20 Table 6- ANTIBODY TITERS to MSP-1$_{42}$ BY ELISA

VALUES INDICATE DILUTION OF SERA WHICH GIVES OD = 1

1/5 Dose	Day 0	Day 14	Day 28	Day 42	Day 84	Day 98
Average	12	312	462	18066	7371	32648
Std Dev	8	191	236	8406	6304	24046
Geo Mean	10	272	412	16440	5749	26626

1/2 DOSE	Day 0	Day 14	Day 28	Day 42	Day 84	Day 98
Average	28	1285	2530	44172	ND	57771
Std Dev	6	1882	2631	21176	ND	24192
Geo Mean	28	636	1762	40744	ND	53569

Full Dose	Day 0	Day 14	Day 28	Day 42	Day 84	Day 98
Average	32	688	990	32461	15914	50053
Std Dev	32	414	306	19307	5650	29991
Geo Mean	22	586	951	28448	14938	42799

40

IFA: The vaccine was also immunogenic as assessed by indirect immunofluorescence titers to malaria parasites.

Table 7- IMMUNOFLUORESCENCE to 3D7 Parasites
Values indicate serum dilution which gives 1+ IFA intensity to methanol-fixed malaria parasites

DAY OF STUDY

1/5 Dose-GROUP 1	Day 0	Day 14	Day 28	Day 42	Day 84	Day 98
Geomean	200	200	200	1393	2111	3676
95% CI				1152	2533	5155
SD	0	0	0	1315	2890	5881
1/2 Dose-GROUP 2	Day 0	Day 14	Day 28	Day 42	Day 84	Day 98
Geomean	200	200	200	2425	ND	5572
95% CI				768	ND	1254
SD	0	0	0	876	ND	1431
Full Dose-GROUP 3	Day 0	Day 14	Day 28	Day 42	Day 84	Day 98
Geomean	200	200	200	3200	3676	8445
95% CI				1536	1882	3967
SD	0	0	0	1753	2147	4525

In addition, cell-mediated immunologic responses were noted in the majority of vaccinated subjects. Peripheral blood mononuclear cells were stimulated with MSP-1 antigen or *P. falciparum* parasitized erythrocytes and proliferation was measured by uptake of ^{3}H-thymidine. Figure 6 shows the results of PBMC proliferation in each of the subjects after each dose of vaccine.

Summary

In this initial clinical trial involving a small number of volunteers, FMP1/ADJUVANT B has been shown to be a safe and highly immunogenic vaccine that elicits both parasite-reactive antibodies and cellular

responses. This study develops a foundation to further test its safety profile and evaluate its efficacy to reduce morbidity and mortality in target populations directly affect by *P. falciparum* malaria.

SAFETY DATA

Table 8: Number of subjects

	TOTAL	Percent	Group1	Group 2	Group 3
Number of subjects planned	15	100%	5	5	5
Subjects or vaccine number not allocated	0	0	0	0	0
Number of subjects enrolled (Total cohort)	15	(100%)	5	5	5

"Reasons for elimination..."
Group 1: FMP1/ADJUVANT B: 1/5th dose
Group 2: FMP1/ADJUVANT B: 1/2 dose
Group 3: FMP1/ADJUVANT B: full dose

Table 9: Demographics : Study population

Sex	N	Mean Age (years)	Min age (years)	Max. Age (years)	S.D. (years)
Female	5	32.2	27	51	10.8
Male	10	34.4	22	52	11.1
Total	15	33.7	22	52	10.7

N = total number of subjects
S.D. = standard deviation

Table 10- Incidence and nature of symptoms reported per dose and per subjectafter vaccination

Dose	Group N	General symptoms		Local symptoms	
		n	%	n	%
By dose					
Dose 1	15	2	13	12	80
Dose 2	15	2	13	11	73
Dose 3	15	1	7	10	67
Overall	45	5	11	33	73

Table 10 Continued

Dose	Group	N	General symptoms		Local symptoms	
			n	%	n	%
By volunteer						
Group 1	5		1	20	4	80
Group 2	5		1	20	4	80
Group 3	5		2	40	5	100

N per dose = Number of documented doses
N per volunteer = Number of volunteers
n per dose = Number of documented doses with at least one symptom
n per subject = Number of subjects with at least one documented dose

Table 11- Incidence of solicited local symptoms including symptoms graded at maximum intensity

Solicited local Symptom	Intensity	Group 1 (N=15)		Group 2 (N=15)		Group 3 (N=15)	
		n	%	n	%	n	%
Pain	Total	8	53.3	11	73.3	14	93.3
	grade"3"	0	0	0	0	0	0
Redness	Total	1	6.7	4	26.7	2	13.3
	> 50 mm/>24h	0	0	0	0	0	0
Swelling	Total	0	0	0	0	0	0
	> 50 mm/>24h	0	0	0	0	0	0

Group 1: FMP1/ADJUVANT B: 1/5th dose
Group 2: FMP1/ADJUVANT B: 1/2 dose
Group 3: FMP1/ADJUVANT B: full dose
N = Total number of documented doses
n = number of documented doses after which there is at least one report of a symptom
%= percentage of documented doses after which there is at least one report of a symptom
Grade "3" = pain preventing normal daily activity and necessitates a corrective therapy
>50mm/>24h = redness or swelling with a diameter of more than 50 mm and persisting for more than 24 hours

Table 12: Subjects reporting solicited local symptoms including symptoms graded at maximum intensity

Solicited local symptom	Intensity	Group 1 (N=5)		Group 2 (N=5)		Group 3 (N=5)	
		n	%	n	%	n	%
Pain	Total	4	80	4	80	5	100
	grade"3"	0	0	0	0	0	0
Redness	Total	1	20	3	60	2	40
	> 50 mm/>24h	0	0	0	0	0	0
Swelling	Total	0	0	0	0	0	0
	> 50 mm/>24h	0	0	0	0	0	0

Group 1: FMP1/ADJUVANT B: 1/5th dose
Group 2: FMP1/ADJUVANT B: 1/2 dose
Group 3: FMP1/ADJUVANT B: full dose
N = Total number of subjects
n = number of subjects reporting at least one report of a symptom
%= percentage of subjects reporting at least symptom
Grade "3" = pain preventing normal daily activity and necessitates a corrective therapy
>50mm/>24h = redness or swelling with a diameter of more than 50 mm and persisting for more than 24 hours

Table 13: Incidence of solicited general symptoms including symptoms graded at maximum intensity and those probably or suspected of being related to vaccination

Symptoms		Group 1 N=15		Group 2 N=15		Group 2 N=15	
		N	%	N	%	N	%
Arthralgia	Total	0	0	0	0	1	6.7
	PB/SU	0	0	0	0	0	0
	PB/SU & Grade "3"	0	0	0	0	0	0
Fever	Total	0	0	0	0	1	6.7
	PB/SU	0	0	0	0	0	0
	PB/SU & Grade "3"	0	0	0	0	0	0
Headache	Total	0	0	0	0	0	0
	PB/SU	0	0	0	0	0	0
	PB/SU & Grade "3"	0	0	0	0	0	0

Table 13 Continued

Symptoms		Group 1 N=15		Group 2 N=15		Group 2 N=15	
		N	%	N	%	N	%
Malaise	Total	1	6.7	1	6.7	1	6.7
	PB/SU	1	6.7	1	6.7	0	0
	PB/SU & Grade "3"	0	0	0	0	0	0
Myalgia	Total	1	6.7	0	0	1	6.7
	PB/SU	1	6.7	0	0	0	0
	PB/SU & Grade "3"	0	0	0	0	0	0
Rash	Total	0	0	0	0	0	0
	PB/SU	0	0	0	0	0	0
	PB/SU & Grade "3"	0	0	0	0	0	0
Dizziness	Total	0	0	0	0	1	6.7
	PB/SU	0	0	0	0	0	0
	PB/SU & Grade "3"	0	0	0	0	0	0
Nausea	Total	0	0	0	0	1	6.7
	PB/SU	0	0	0	0	0	0
	PB/SU & Grade "3"	0	0	0	0	0	0

Group 1: FMP1/ADJUVANT B: 1/5th dose
Group 2: FMP1/ADJUVANT B: * dose
Group 3: FMP1/ADJUVANT B: full dose
N = Total number of documented doses.
n= number of documented doses after which there is at least one report of a symptom.
%= percentage of documented doses after which there is at least one report of a symptom.
Grade "3" = Adverse experience which prevents normal everyday activities and necessitates a corrective therapy.
PB /SU= Probably or suspected of an association. -

Table 14: Subjects reporting solicited general symptoms including symptoms graded at maximum intensity and those probably or suspected of being related to vaccination

Symptoms		Group 1 (1/5 dose) N=5 N	%	Group 2 (1/2 dose) N=5 N	%	(Full dose) Group 2 N=5 N	%
Arthralgia	Total	0	0	0	0	1	20
	PB/SU	0	0	0	0	0	0
	PB/SU & Grade "3"	0	0	0	0	0	0
Fever	Total	0	0	0	0	1	20
	PB/SU	0	0	0	0	0	0
	PB/SU & Grade "3"	0	0	0	0	0	0
Headache	Total	0	0	0	0	0	0
	PB/SU	0	0	0	0	0	0
	PB/SU & Grade "3"	0	0	0	0	0	0
Fatigue	Total	1	20	1	20	1	20
	PB/SU	1	20	1	20	0	0
	PB/SU & Grade "3"	0	0	0	0	0	0
Myalgia	Total	1	20	0	0	1	20
	PB/SU 1	20	0	0	0	0	0
	PB/SU & Grade "3"	0	0	0	0	0	0
Rash	Total	0	0	0	0	0	0
	PB/SU	0	0	0	0	0	0
	PB/SU & Grade "3"	0	0	0	0	0	0
Dizziness	Total	0	0	0	0	1	20
	PB/SU	0	0	0	0	0	0
	PB/SU & Grade "3"	0	0	0	0	0	0
Nausea	Total	0	0	0	0	1	20
	PB/SU	0	0	0	0	0	0
	PB/SU & Grade "3"	0	0	0	0	0	0

N = Total number of subjects.
n= number of subject reporting at least one report of a symptom.
%= percentage of subject reporting at least one report of a symptom.
Grade "3" = Adverse experience which prevents normal everyday activities and necessitates a corrective therapy.
PB /SU= Probably or suspected of an association.

Example 5

Three rabbits per immunization group were
vaccinated 4 times at 3-week intervals with 50 μg FMP1
5 (3D7) in Montanide adjuvant, subcutaneously, or with
FMP1 in ADJUVANT B, intramuscularly. Two negative
control rabbits per group were immunized with each
adjuvant alone. A final control rabbit was immunized
with reduced and alkylated MSP1$_{42}$ (3D7) in Montanide.
10 Each rabbit was bled from the ear vein 2 weeks
following each immunization. Following the fourth
immunization the rabbits were ex-sanguinated from the
heart and the sera from these rabbits was analyzed by
MSP1 (3D7)-specific antigen ELISA's.
15   The sera were analyzed by MSP1 (3D7)-specific
ELISA and by kinetic ELISA. The MSP1-specific capture
antigen was diluted in PBS at pH 7.4 and coated at
0.4pmoles/well overnight at 4°C and the wells were
blocked with CaseinBlock (Pierce). Sera were first
20 diluted by 1:25 and then followed by two-fold serial
dilutions down the plate up to 1.6 x 10^6 fold. Sera
were reacted for 1 hour at room temperature, followed
by reaction with alkaline phosphatase-conjugated goat
anti-rabbit IgG (H&L)(Promega) diluted 1:5,000 in
25 CaseinBlock for 1 hour at room temperature. Detection
of p-nitrophenyl phosphate substrate conversion to
product was measured at 60 minutes. The data are
reported as the average of triplicate values plotted
from the titration curve measured at OD$_{405}$.
30   Immunization with FMP1 in Montanide and FMP1 in
ADJUVANT B induces high MSP1$_{42}$ specific antibody titers
following the second immunization. The geometric mean
of the post fourth immunization MSP1$_{42}$ specific
antibody titers induced by immunization with
35 FMP1/ADJUVANT B and FMP1/Montanide were 1: 363,000 and

1:182,000, respectively. Neither adjuvant/antigen combination substantially boosted the MSP1$_{42}$ specific antibody titers after a third and fourth immunization.

5

DISCUSSION

Here we describe the development, fermentation, expression, purification and characterization of a safe and immunogenic recombinant MSP-1$_{42}$ that is

10 suitable for production of antibodies and for use in diagnostic assays and as a potential vaccine.

MSP-1$_{42}$ which was derived from recombinant *E. coli* was highly purified and met all FDA standards necessary for testing safety and immunogenicity in

15 humans. Endotoxin levels were significantly below the FDA acceptable levels (FDA; 350 EU/dose/70kg human, Our Process; 9.14 EU/dose/70kg human). Residual levels of all chemicals used in the purification process were quantified and determined to be within levels set as

20 production specifications. Although the MSP-1$_{42}$ comprised greater than 95% of the protein in the final product, it was not homogeneous due to proteolysis at the C-terminal end of the protein. This proteolysis appeared to occur during expression in the *E. coli*

25 host because long term studies showed that the antigen was stable when stored at -80°C for 18 months, revealing little change in the Coomassie Blue staining pattern or mAb reaction patterns in immunoblots (Figure 3).

30 Previous studies have shown that the induction of antibody responses to epitopes on MSP-1$_{19}$ correlate with clinical immunity to malaria (Egan et al., 1996, J. Infect. Dis. 173, 765-769), suggesting that induction of such responses depends on correctly

35 forming the disulfide-dependent conformational

epitopes present within the MSP-1_{19} portion of MSP-1_{42}.
This conclusion is further supported by the
observation that the only MSP-1_{19} specific mAbs capable
of inhibition merozoite invasion react with
5   conformational epitopes (Burghaus et al., 1994, Mol.
Biochem. Parasitol. 64, 165-169). Recombinant MSP-1_{42}
has correct structure because it reacted with all of
the MSP-1_{19} specific mAbs we used; these were raised
against malaria parasites and included functional mAbs
10   classified as growth or invasion inhibitory (mAb
12.10, 12.8) (Blackman et al., 1990, supra) and
blocking inhibitory mAbs (mAb 7.5, 2.2, 1E1) (Guevara
et al., 1997, J. Exp. Med. 186, 1689-1699).

These data support the continued use of bacterial
15   systems for expressing soluble malaria antigens that
contain conformational epitopes as potential vaccine
candidates.

In addition, FMP1/ADJUVANT B was highly
immunogenic in the Rhesus monkeys as well as Balb/C
20   mice. After three immunizations the geometric mean IFA
antibody titers induced in Rhesus monkeys exceeded
1:36,000 and the geometric mean ELISA titer exceed
26,000 OD/min (Figure 4); these titers did not change
with further immunization. FMP1/ADJUVANT B induced
25   approximately six times more MSP-1 specific antibodies
than FMP1/Alum (Figure 4). MSP-1 specific antibodies
induced by FMP1 were predominantly against MSP-1_{42} and
MSP-1_{19} compared to MSP-1_{33} (not shown). This result,
when taken in combination with FMP1's ability to
30   induce high titer antibodies that react with schizont-
infected erythrocytes and it's ability to induce
invasion inhibitory antibodies (Figure 5 and Table 1)
further indicates that it has correct structure.
Currently, the only malaria vaccine that has
35   reproducibly protected human volunteers is the

RTS,S/ADJUVANT B vaccine (Kester et al., 2001, J. Infect. Dis. 183, 640-647). When combined with ADJUVANT B, FMP1 was highly immunogenic and caused no adverse biochemical or local reactions in mice.

5

10

15

20

25

30